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## **Deciphering the Neuroprotective Potential of Interleukin-1 $\beta$ (IL-1 $\beta$ ) using in vitro Murine Models of Oxidative Stress**

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## **Abstract**

Interleukin-1 $\beta$  (IL-1 $\beta$ ), a key cytokine driving neuroinflammation in the Central Nervous System (CNS), is enhanced in many neurological diseases/disorders, including but not limited to ischemic stroke, Parkinson's disease, Amyotrophic Lateral Sclerosis, Huntington's disease and Alzheimer's disease. The dominant view is that IL-1 $\beta$  contributes to and/or sustains pathophysiological processes. However, other studies demonstrate that IL-1 $\beta$  can play an important role in neural protection and repair. It may do so by modifying astrocyte behavior. Indeed, studies from our laboratory demonstrate that IL-1 $\beta$  increases the synthesis and release of the antioxidant molecule glutathione (GSH) from astrocytes and that IL-1 $\beta$ -treated astrocytes show increased resistance to oxidative stress [1], a pathological process that leads to neural damage in the above-mentioned maladies. Given this, the overall goal of this thesis was to study the protective potential of IL-1 $\beta$  against oxidant injury in astrocytes, neurons and astrocyte and neurons in co-culture.

We confirmed that IL-1 $\beta$  mediates an increase in extracellular GSH levels in cortical wild-type astrocytic (as shown by our laboratory earlier [1]) and mixed cultures but not neuronal culture. IL-1 $\beta$ -mediated GSH enhancement rendered protection to mixed culture against oxidative stress induced by the stressor tert-butyl hydroperoxide (t-BOOH). GSH production and the resultant protection were blocked by the inhibition of GSH transport through Mrp1. Additionally, IL-1 $\beta$  failed to increase GSH or to provide protection against t-BOOH toxicity in chimeric

cultures made using *il1r1* null mutant astrocytes (lacking a functional IL-1R1 receptor) indicating the necessity of astrocytic signaling for the observed phenomenon. Overall, these findings suggest that under certain conditions IL-1 $\beta$  may be an important stimulus for GSH production and consequent neuroprotection through astrocyte-specific signaling.

Deciphering the Neuroprotective Potential of Interleukin-1 $\beta$  (IL-1 $\beta$ ) using *in vitro*  
Murine Models of Oxidative Stress

by

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B.S., University of Mumbai, 2010

M.S., University of Mumbai, 2012

Dissertation

Submitted in partial fulfillment of the requirements for the degree of  
Master of Science in Biology.

Syracuse University

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<b>Table of Contents</b>	<b>Pg no.</b>
List of Tables	ix
List of Figures	x
List of Abbreviations	xii
<b>Chapter 1. Introduction</b>	<b>1</b>
1.1. IL-1 $\beta$ and Neuroinflammation	1
1.1.1. IL-1 family of cytokines	1
1.1.2. IL-1 $\beta$	2
1.1.3. IL-1 $\beta$ Signaling	3
1.1.4. IL-1Ra	4
1.1.5. IL-1 $\beta$ : Beneficial or inimical?	4
1.2. Oxidative Stress and Neurodegeneration in the CNS	6
1.2.1. Reactive species in the brain	6
1.2.2. Oxidative stress in the brain	7
1.2.3. Antioxidants in the brain	10
1.3. Glutathione	12
1.3.1. Glutathione synthesis, function and import	12
1.3.2. Glutathione in the CNS	16
1.3.3. IL-1 $\beta$ and GSH synthesis in the CNS	17
1.4. Specific Aim	18
<b>Chapter 2. Materials and Methods</b>	<b>19</b>
2.1. Experimental Animals	19



2.2. Cell Culture	19
2.3. IL-1 $\beta$ Treatment	21
2.4. Measurement of GSH	22
2.5. Drug Exposure	23
2.6. Measurement of Cell Death and Viability	24
2.7. Propidium Iodide Staining	25
2.8. Statistical Analyses	25
<b>Chapter 3. Results</b>	<b>26</b>
3.1. Astrocytes	26
3.2. Neurons	27
3.3. Mixed culture	28
3.4. Neurons + GEE	29
<b>Chapter 4. Discussion</b>	<b>45</b>
<b>Chapter 5. Future Directions</b>	<b>54</b>
<b>Bibiography</b>	<b>56</b>

<b>List of Tables</b>	<b>Pg no.</b>
Table 1: The IL-1 superfamily of cytokines.	2

<b>List of Figures</b>	<b>Pg no.</b>
Figure 1: Glutathione synthesis	12
Figure 2: Glutathione reactions	14
Figure 3: IL-1 $\beta$ pre-treatment increases the GSH status of astrocytes.	30
Figure 4: IL-1 $\beta$ pre-treatment protects pure astrocytes against t-BOOH-mediated cell death.	31
Figure 5: Increase in extracellular GSH and protection against t- BOOH-mediated cell death in astrocytes is purely due to IL- 1 $\beta$ signaling through IL-1R1.	32
Figure 6: Attenuation of astrocytic GSH extrusion does not affect the cellular viability upon t-BOOH challenge.	33
Figure 7: IL-1 $\beta$ pre-treatment does not change the GSH status of neurons.	34
Figure 8: t-BOOH causes cell death in a concentration-dependent manner in neurons.	35
Figure 9: IL-1 $\beta$ pre-treatment does not protect neurons against t-BOOH-toxicity.	36
Figure 10: Increasing the concentrations of IL-1 $\beta$ pre-treatment does not protect pure neurons against t-BOOH-mediated cell death.	37
Figure 11: IL-1 $\beta$ pre-treatment increases the GSH release from mixed cultures.	38

Figure 12: IL-1 $\beta$ pre-treatment protects mixed cultures against t-BOOH toxicity.	39
Figure 13: IL-1 $\beta$ pre-treatment of mixed culture protects both astrocytes and neurons.	40
Figure 14: Increase in extracellular GSH in chimeric culture is astrocyte IL-1R1-dependent.	41
Figure 15: IL-1 $\beta$ pre-treatment protects neurons plated on <i>il1r1</i> $+/+$ but not <i>il1r1</i> $-/-$ astrocytes in chimeric mixed culture against t-BOOH-mediated cell death.	42
Figure 16: Attenuation of astrocytic GSH extrusion decreases cellular viability in mixed culture upon t-BOOH treatment.	43
Figure 17: Supplementation of pure neurons with GSH-Ethyl Ester (GEE) does not affect the cell viability upon t-BOOH treatment.	44

## **List of Abbreviations**

8-OHdG	8-hydroxy deoxyguanosine
A $\beta$	Amyloid $\beta$ -peptide
AD	Alzheimer's Disease
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activating protein 1
AraC	$\beta$ -D-arabinofuranoside
ARE	Antioxidant response element
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BGS	Bovine growth serum
BSS	Balanced salt solution
CNS	Central nervous system
CS	Calf serum
Cys	Cysteine
Cyss	Cystine
DMEM	Dulbecco's modified eagle medium
E15	Embryonic day 15
FBS	Fetal bovine serum
GGT1	$\gamma$ -glutamyl transpeptidase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
ICE	Interleukin-1-converting enzyme
IL-1 $\alpha$	Interleukin-1 $\alpha$
IL-1 $\beta$	Interleukin-1 $\beta$
IL-1RI	Interleukin-1 Receptor type I
IL-1RII	Interleukin-1 Receptor type II
IRAK	IL-1R-associated kinase
JNK	c-Jun N-terminal

KO	Knockout
LDH	Lactate dehydrogenase
MAP	Mitogen activated protein
MnSOD	Manganese superoxide dismutase
Mrp1	Multidrug resistance protein 1
MS	Multiple sclerosis or media stock
MTT	Thiazolyl blue tetrazolium bromide
MyD88	Myeloid differentiation primary response gene 88
NAC	N-acetyl cystine
NF- $\kappa$ B	Nuclear factor $\kappa$ B
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
Nrf2	Nuclear factor erythroid derived 2-related protein
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RNOS	Reactive nitrogen oxygen species
t-BOOH	Tert-butyl hydroperoxide
TNF	Tumor necrosis factor
WT	Wild-type

## **Chapter 1. Introduction**

### **1.1 IL-1 $\beta$ and Neuroinflammation**

Inflammation in the CNS (a.k.a. neuroinflammation) and elsewhere in the body is mediated by a number of soluble factors, including a group of secreted molecules termed cytokines. Inflammatory cytokines can contribute to either acute inflammation which is generally viewed as a protective/repairative response [2-4] or to chronic inflammation which is commonly considered deleterious to tissues harmful to impaired CNS [5-9]. Some cytokines, such as IL-1 $\beta$ , contribute to both [10-12]. This dissertation is directed at studying the potential protective effects of IL-1 $\beta$  against oxidant stress in central nervous system tissue neuroinflammation.

#### **1.1.1. IL-1 family of cytokines**

IL-1 $\beta$  is a member of the canonical IL-1 family that consists of two agonists IL-1 $\alpha$  and IL-1 $\beta$ , a naturally occurring receptor antagonist IL-1Ra, and two receptors (IL-1RI and IL-1RII) [13-15]. Currently this family has been extended with additional members added based on conservation of key amino acid sequences and similar three-dimensional structure [9, 16-18]. All ligands and receptors are expressed in the healthy CNS at low levels [19-21]. IL-1 has been shown to be produced by microglia [22, 23], astrocytes [24, 25], oligodendrocytes [26], and neurons [20, 27]. These same cell types are also capable of responding to the

cytokine [26, 28-30]. Further discussion will be limited to IL-1 $\beta$ , the key cytokine discussed in this thesis.

**Table 1: The IL-1 superfamily of cytokines** (Figure adapted from [31]).

New name	Other Name	Property
IL-1F1	IL-1 $\alpha$	Agonist
IL-1F2	IL-1 $\beta$	Agonist
IL-1F3	IL-1Ra	Receptor antagonist
IL-1F4	IL-18; IFN- $\gamma$ -inducing factor	Agonist
IL-1F5	FIL1 $\delta$	Anti-inflammatory
IL-1F6	FIL-1 $\epsilon$	Agonist
IL-1F7	IL-1H4, IL-1 $\zeta$	Anti-inflammatory
IL-1F8	IL-1H2	Agonist
IL-1F9	IL-1 $\epsilon$	Agonist
IL-1F10	IL-1Hy2	Receptor antagonist?
IL-1F11	IL-33	Agonist

### 1.1.2 IL-1 $\beta$

IL-1 $\beta$  is truly a pleiotropic cytokine and affects the innate as well as the acquired immune systems. The agonists are synthesized as precursor molecules without a signal peptide. After the removal of N-terminal amino acids by proteases, the



resulting peptides are called “mature” forms (See review [32]). The 31 kDa precursor form of IL-1 $\beta$  is biologically inactive and requires cleavage by a specific intracellular cysteine protease called IL-1 $\beta$  converting enzyme (ICE) or caspase 1 [33]. The mature form of IL-1 $\beta$  is a 17.5 kDa protein. Although ICE cleaves the pro-ligands intracellularly, other proteases such as proteinase-3 can process the IL-1 $\beta$  precursor extracellularly into a mature ligand [34].

### **1.1.3 IL-1 $\beta$ Signaling**

IL-1 $\beta$  can bind to two receptors, the type I and type II, referred to as IL-1RI and IL-1RII, respectively. Each has a large extracellular domain composed of a series of three immunoglobulin (Ig)-like motifs; however, only IL-1RI possesses a large intracellular Toll-like/IL-1 receptor (TIR) domain, which participates in the intracellular downstream signaling. At the surface, IL-1 $\beta$  forms a dimer with IL-1RI and induces a conformational change: the Ig-like domains hook around the bound IL-1 molecule [35]. Following this, another protein known as the IL-1 receptor accessory protein or IL-1RAcP binds to the IL-1RI/IL-1 complex, bringing together their Toll-like/IL-1 receptor (TIR) domains [36, 37]. After the recruitment of the IL-1/IL-1RI/IL-1RAcP complex, MyD88 (myeloid differentiation factor 88), a signaling molecule attaches to the complex [38]. This leads to the association with IL-1 receptor associated kinases (IRAK)-1 and IRAK-4 [37]. IRAK-1 and IRAK-4 when activated, can mediate downstream signaling events, which can result in the activation of the NF- $\kappa$  B pathway [39-43], the p38

mitogen-activated protein (MAP) kinase pathway [44], the c-Jun N-terminal kinase (JNK) pathway [45] and the Src family of tyrosine kinases [46-48].

IL-1RII, which lacks the intracellular signaling domain, is thought to function as a “decoy” receptor or negative regulator of IL-1 $\beta$  signaling [31]. The binding of IL-1RII to IL-1 $\beta$  at the cell prevents the ability of the IL-1 $\beta$  to form a complex with the type I receptor and the accessory protein [49, 50]. Another proposed function of the decoy receptor is to form a trimeric complex of the ligand with the type II receptor and the accessory protein [51, 52].

#### **1.1.4 IL-1Ra**

IL-1Ra is a naturally occurring IL-1RI antagonist. It is a specific receptor antagonist for IL-1 $\alpha$  and IL-1 $\beta$ . The binding of IL-1RI to IL-1RAcP is required for intracellular signaling and it is this association that is blocked by IL-1Ra [53-55].

#### **1.1.5. IL-1 $\beta$ : Beneficial or inimical?**

Interestingly, IL-1 $\alpha$ , IL-1 $\beta$ , and IL-1RI knockout mice show no gross physiological or developmental defects [56, 57]. And IL-1 $\beta$  alone, in the absence of additional CNS impairment, is not neurotoxic [2-4]. Despite this, many studies suggest that IL-1 $\beta$  is harmful to impaired CNS [5-9]. However, studies also suggest it to be neuroprotective [58-62]. Whether IL-1 $\beta$  is beneficial or inimical appears to depend on the concentration of the cytokine, status of surrounding cells and timing of release [59, 63-65]. The following is a description of a few paradigms in

which the laboratory has been studying the role of IL-1 $\beta$  that serves to illustrate the dichotomy described above.

**IL-1 $\beta$  and seizures:** The incidence of convulsive seizures induced by the chemoconvulsant pentylenetetrazol (PTZ) increases in null mutant mouse lines lacking the IL-1 $\beta$  ligand or the receptor IL-1R1 as compared to their respective wild-type littermate controls [66]. These results suggest that IL-1 $\beta$  serves as an endogenous modulator to suppress seizure generation.

**IL-1 $\beta$  and cerebral ischemia:** IL-1R1 null mutant mice are less susceptible than wild-type control mice to focal cerebral ischemic damage induced by reversible MCAO [67]. These studies complemented previous published work in experimental ischemia models showing that animals deficient in ICE/caspase 1 had a reduction in IL-1 $\beta$  levels and a concomitant reduction in ischemic infarct volumes as compared to wild-type mice [68, 69]. Additionally, administration of an IL-1 $\beta$  neutralizing antibody improved outcomes following ischemia in rats [70]. Finally, intracerebroventricular administration and genetic overexpression of IL-1Ra, the endogenous antagonist, before MCAO, is protective and reduces infarct size [3, 71-74]. Moreover, we have shown that IL-1 $\beta$  treatment enhances neuronal injury in a variety of neuronal injury models associated with energy deprivation [67, 75]. These studies demonstrate a predominantly negative effect of IL-1 $\beta$  in the setting of cerebral ischemia.

**IL-1 $\beta$  and oxidative stress:** Oxidative stress is involved in conditions of critical injury to neurons and glia in the CNS. Reactive nitrogen and oxygen species

(RNOS) are part of the cascade that eventually lead to cell death [76]. A correlation between accumulation of ROS and an increase in IL-1 $\beta$  during oxidative stress has been demonstrated [77-79], suggesting RNOS production could increase IL-1 $\beta$  levels. In support, plasminogen-induced IL-1 $\beta$  production from microglia is reduced in the presence of the antioxidants trolox or N-acetylcysteine (NAC) [80]. But of what consequence is this increase? We have found that IL-1 $\beta$  mediates a time-dependent increase in extracellular GSH levels production from cortical astrocyte cultures, suggesting both enhanced synthesis and export. Moreover, IL-1 $\beta$  treatment prevented the increase in ROS produced in astrocytes following exposure to peroxide. Further, the toxicity associated with this exposure was significantly attenuated following treatment with IL-1 $\beta$  [1]. These data point to a positive, protective role for IL-1 $\beta$  against oxidative stress. This result is of particular importance given the contributory role of oxidative stress to neuronal injury in the CNS disease/disorders.

## **1.2. Oxidative Stress and Neurodegeneration in the CNS**

### **1.2.1. Reactive species in the brain**

The human brain accounts for only 2% of the body weight but it processes 20% of basal O<sub>2</sub> consumption [81]. During aerobic energy metabolism, electrons are transported via the electron carriers in the mitochondria to generate ATP and oxygen acts a final acceptor of electrons and gets reduced to form water [82]. A substantial concentration of oxygen gets partially reduced to form reactive oxygen species (ROS). Over 90% of ROS is produced in the mitochondria [83],

with superoxide ( $O_2^-$ ) making up a substantial fraction. An important enzymatic source of superoxide is nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) which catalyzes a one electron reduction of molecular oxygen to form  $\cdot O_2^-$  [84].

$O_2^-$  may undergo Haber–Weiss reaction ( $\cdot O_2^- + H_2O_2 \rightarrow \cdot HO + O_2 + HO^-$ ), generating more toxic radicals. Iron salts and metal ions present at low levels in biological systems, serve as catalysts for this pathway [85]. Trace elements, iron, aluminum, mercury, and copper are capable of stimulating free radical generation. Iron is an important source of reactive species through its participation in the Fenton reaction. Studies have demonstrated that  $H_2O_2$  can oxidize  $Fe^{2+}$  to produce hydroxide ( $OH^-$ ) and the highly reactive hydroxyl radical ( $OH^\cdot$ ), and this constitutes the Fenton reaction [86]. The highly toxic hydroxyl radical is very reactive with lipids [87].

Additional reactive species derived from nitrogen (Reactive nitrogen species or RNS) include nitric oxide (NO) [88], peroxynitrite ( $ONOO^-$ ), nitrogen dioxide ( $NO_2$ ), and nitroxyl (HNO). These reactive species are all capable of causing oxidative modifications of lipids, proteins, or DNA.

### **1.2.2. Oxidative stress in the brain**

Under physiological conditions, RNOS generation and the endogenous antioxidant systems are well-balanced. However, if the level of RNOS increases or the level of antioxidants decreases, this balance will be disturbed leading to

oxidative stress in the CNS. Helmut Sies defined oxidative stress as “a disturbance in the pro-oxidant - antioxidant balance in favor of the former” [89]. Subsequent research has shed some light on redox signaling and its consequence on the oxidative status of cells and this has redefined oxidative stress as “a disruption of redox signaling and control.” (reviewed in [90]).

Oxidative reactions are key players in aging and major disease processes, including but not limited to cancer, cardiovascular and pulmonary diseases, diabetes and neurodegeneration [91-94]. The oxidation of biomolecules such as DNA and proteins and the peroxidation of lipids can initiate deleterious processes in a variety of acute and chronic neurological disorders/diseases [95-102].

Lipid peroxidation is the oxidation of lipids that is a crucial step in the pathogenesis of several diseases. Several lipid molecules containing double bond(s) can be oxidized under appropriate conditions [103]. The mechanisms inducing lipid peroxidation are complex. Polyunsaturated fatty acid is known to be rich in the membranes of neurons. Lipid peroxidation is the result of attack by radicals on the double bond of unsaturated fatty acids, such as arachidonic acid, to give rise to highly reactive lipid peroxy radicals that initiate a chain reaction of further attacks on other unsaturated fatty acids. Lipid peroxidation is a self-propelling chain-reaction and the initial oxidation of a few molecules can result in magnified tissue damage. Primary peroxidation products of fatty acid oxidation are hydroperoxides in which double bond(s) may have moved or/and changed configuration. These products may be converted into secondary peroxidation

products [104]. Lipid peroxides and lipid-derived aldehyde adduct proteins are players in the cascade of neurodegeneration [105]. Two main products of the peroxidation reaction are: malondialdehyde (MDA) and, in particular, 4-hydroxy-2-nonenal (4-HNE) that are also used as biomarkers of oxidative stress [106].

Proteins are another target of reactive species. Protein oxidation has also been studied in disorders and diseases involving oxidative stress. Covalent modification of protein takes place in the oxidation reaction and is induced either by the direct reactions with reactive species or indirectly by the secondary by-products of oxidative stress [107]. The amino acids cysteine and methionine are most vulnerable to oxidative changes due to the sulfur groups. Protein carbonyl content (PCC) is the most widely used marker of protein oxidation in cells [108].

Finally, oxidation of DNA is another consequence of release or imbalance of reactive species in the cell. Irrespective of their origin, RNOS may interact with DNA, leading to modification of the bases. Pyrimidine radicals can occur as a result of oxidative damage yielding numerous products by several mechanisms [109]. Hydroxyl radical reacts with DNA by addition to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and moieties of ribose sugar in the backbone [110]. Hydroxyl radical can also induce the formation of DNA-protein cross-links. Thymine-tyrosine cross-link is the product from the formation of a covalent bond between the methyl group of the thymine and the third carbon of the tyrosine ring [110]. Guanine, a purine, can be

attacked by hydroxyl radicals as well as singlet oxygen leading to the formation of 8-oxo-7,8-dihydroguanine, a typical marker of DNA oxidation [111].

Neuronal cell death is a complex process and involves multiple mechanisms. Oxidative stress may not be the cause of neuronal cell death, *per se*, but contribute to the cascade eventually leading to cell death [76]. RNOS are secondary to disorders and diseases in the CNS and can damage neurons and promote the release of excitatory amino acids, generating a harmful chain of events [112]. They can also initiate self-propelling reactions leading to the formation of more complex reactive species, the presence of which may be detrimental to the cells.

### **1.2.3. Antioxidants in the brain**

The brain has a wide spectrum of antioxidants which may act enzymatically (superoxide dismutase, catalase, glutathione peroxidase) or non-enzymatically (Vitamin E, Vitamin C and glutathione) to detoxify RNOS [113, 114]. A short review of each follows:

Vitamin C or Ascorbic Acid: Ascorbic acid is a potent water soluble dietary antioxidant with the ability of neutralizing a spectrum of reactive oxygen species such as hydroxyl, alkoxyl, peroxy, superoxide anion, hydroperoxyl radicals and reactive nitrogen radicals such as nitrogen dioxide, nitroxide, peroxynitrite at very low concentrations [115]. Vitamin C has been shown to interact with free,



catalytically active metal ions producing hydroxyl and alkoxyl *in vitro*. It is controversial whether this mechanism occurs *in vivo* [115].

Vitamin E: Unlike vitamin C which is water soluble, Vitamin E is a fat soluble dietary antioxidant [116]. It is a potent scavenger of peroxy radical and controls the propagation of free radical damage in biological membranes [117]. More information can be found in this comprehensive review [118].

Super oxide dismutase (SOD): SOD catalyzes the conversion of two moles of  $\cdot\text{O}_2^-$  to  $\text{H}_2\text{O}_2$ , which is itself converted to water by catalase and glutathione peroxidase. There are four known SODs. Copper (Cu) and zinc (Zn) SODs (CuZn) are present in the cytosol and manganese (Mn) SOD is mitochondrial. An extracellular SOD (EC SOD) has also been identified [84].

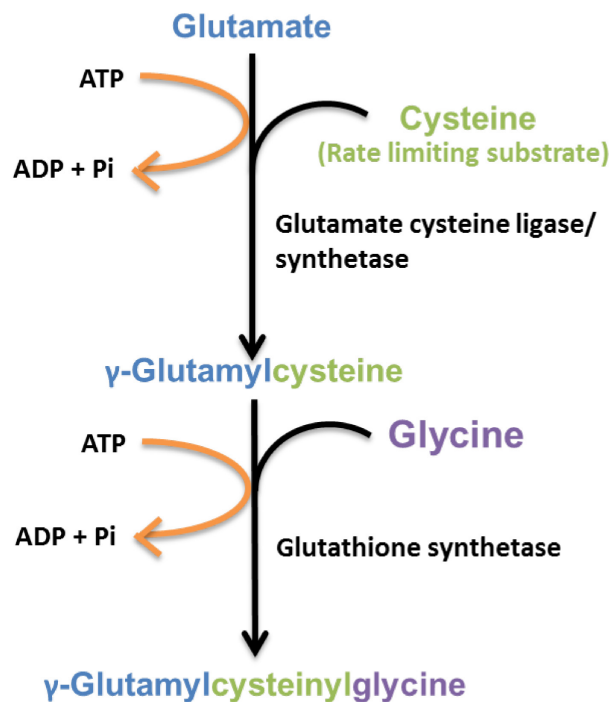
Catalase: Catalases are ubiquitous enzymes that prevent cell oxidative damage by degrading hydrogen peroxide to water and oxygen ( $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ ) with high efficiency [119].

Glutathione: Of particular interest for this thesis is glutathione (GSH). GSH is not only an antioxidant but is also required for the maintenance of cellular health and proliferation [120]. The details of glutathione metabolism, reactions, transport and import are discussed in detail in the next section.

### 1.3. Glutathione

#### 1.3.1 Glutathione synthesis, function transport and import.

GSH Synthesis: GSH is a tripeptide thiol comprised of cysteine, glutamate and glycine ( $\gamma$ -L-glutamyl-L- cysteinylglycine). It is believed to be the most abundant antioxidant in the central nervous system (CNS) [121, 122]. The cellular thiol is present in concentrations up to 12 mM in mammalian cells [121]. GSH is a major redox regulator in the cells [123]. GSH synthesis takes place via a sequential two-step enzymatic reaction [113, 124].

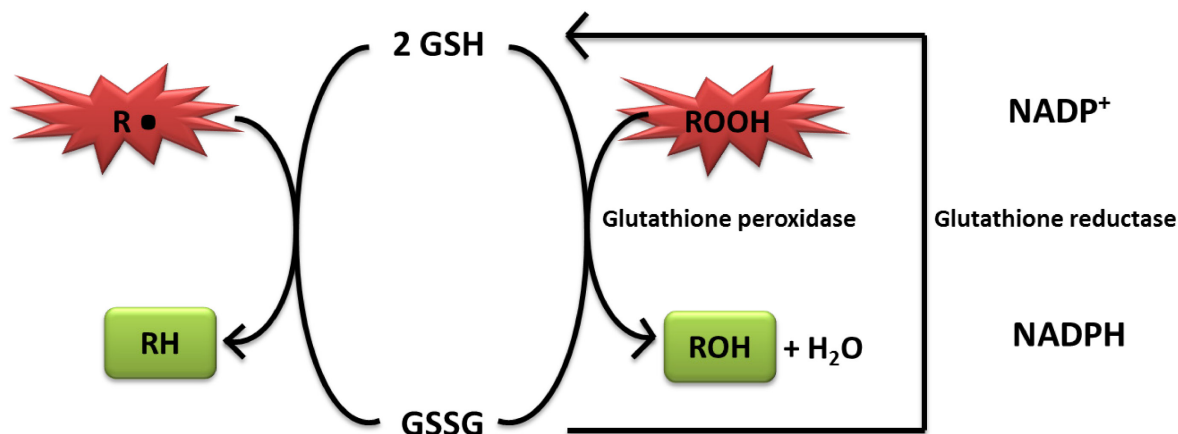


**Figure 1: Glutathione synthesis**

(Adapted from <http://www.vitamor.com/immunopro/glutathion.htm>)

GSH maintains the thiol redox potential in cells by keeping sulfhydryl groups of cytosolic proteins in the reduced form. It has important functions as an antioxidant, a detoxification reagent for xenobiotics and a storage and transport form of the amino acid cysteine [121, 125]. GSH can act either as a non-enzymatic scavenger or a co-factor (an electron donor) for the antioxidant enzyme glutathione peroxidase (GPx) (reviewed in [96, 126]). GSH reacts directly with radicals in non-enzymatic reactions and is the electron donor in the reduction of peroxides catalyzed by glutathione peroxidase. GPx is a selenocysteine-containing enzymes and exists as a group of isozymes. A selenium- independent GPx has been identified [127-129]. Glutathione peroxidase 1 (GPx1) is the most abundant version and it has been found to localize primarily in neuroglial cells, in which the peroxidase activity is tenfold higher than in neurons [130].

Upon oxidation, GSH is converted to glutathione disulfide (GSSG). Regeneration of GSH from GSSG takes places in a reaction catalyzed by the enzyme glutathione reductase (GR) (reviewed in [96]). GR uses NADPH for the transfer of a reduction equivalent.



**Figure 2. Glutathione reactions** (Figure adapted from [131]).

GSH S-transferases catalyze the conjugation of GSH to a variety of toxic reaction products formed by the ROS-dependent peroxidation of intracellular molecules [132].

Transport of GSH: GSH is synthesized intracellularly but there is a requirement for glutathione not only in the cytoplasm, but in the extracellular milieu. The transport of glutathione to the different organelles as well as across the cell membrane has been biochemically demonstrated by several studies; however, the molecular identity of the transporters has not been completely deciphered [133]. GSH homeostasis across the cell membrane requires glutathione transport. Studies from the 1970s proposed the presence of GSH specific carriers that mediate efflux into plasma and other extracellular fluids [134]; however, the direct uptake of GSH molecule into the cells is a subject of debate[135].

The multi drug resistance protein (Mrp) family of transporters, which is a subclass of the ATP-Binding Cassette (ABC) transporter superfamily, have been found to transport GSH across the membranes [136]. The Mrp proteins mediate not only GSH export, but also export of its oxidized derivatives such as GSSG, S-nitrosoglutathione (GS-NO), and glutathione–metal complexes, as well as other glutathione S-conjugates [137]. The Mrp family has five proteins (Mrp1-5). Mrp1 is a transporter that is present in all tissues. Several mechanisms have been proposed with respect to transport of GSH: a) GSH itself is a substrate for the Mrp transporters, b) GSH is co-transported with another substrate and c) GSH transport is stimulated by some compounds which are not transported themselves (reviewed in [137]). The  $K_m$  for GSSG and GSH transport through mammalian Mrp1 are 100  $\mu$ M and 1-10 mM respectively [133].

Uptake of GSH: The uptake of intact GSH into mammalian cells has been much debated [138]. Biochemical experiments have shown the presence of  $\text{Na}^+$ -dependent and  $\text{Na}^+$ -independent glutathione transport systems in brain cells [139]. Studies have also shown the import of products of GSH degradation. Degradation of GSH by  $\gamma$ -GT is followed by uptake of the constituent products and re-synthesis of GSH.

As mentioned, GSH is an important antioxidant/thiol reagent for maintenance of cellular homeostasis for all cell types. However, as the focus of this thesis is on astrocyte and neurons, I will restrict comments to these cell types when describing its role in the CNS.

### 1.3.2 Glutathione in the CNS

The amount of GSx (GSH+GSSG) in neurons is almost exclusively GSH with just 2.5% being GSSG and application of cysteine in the absence or the presence of glycine and/or glutamine has resulted in a doubling of the neuronal glutathione content [140]. Astrocytes, on the other hand, prefer to use cystine instead of cysteine for GSH synthesis and are very efficient in transporting the GSH to the extracellular space [141]. The GSH released from astrocytes is partially used as substrate of the ectoenzyme  $\gamma$ -Glutamyl transpeptidase ( $\gamma$ GT) [142]. The dipeptides CysGly (product of the reaction catalyzed by  $\gamma$ GT) and  $\gamma$ GluCys can serve as precursors for glutathione synthesis in the neurons [140].

In general, neuronal GSH levels are lower than astrocytic GSH [124] rendering them more susceptible to oxidative injury. It was found that neurons co-cultured with astrocytes have higher levels of glutathione than neurons cultured alone, suggesting that astrocytes enhance the neuron antioxidant levels [143].

Astrocytes export a substantial amount of their GSH (10%/hr) into the extracellular space via the Mrp1 transporter [144]. Evidence suggests that it is broken down into dipeptides CysGly and  $\gamma$ GluCys, which can serve as precursors for glutathione synthesis in the neurons. Cysteine is a rate-limiting substrate for GSH synthesis and it has been shown to be derived from astrocytes for the neuronal GSH homeostasis [145, 146].

### 1.3.3 IL-1 $\beta$ and GSH Synthesis in the CNS

System  $x_c^-$  functions in a  $\text{Na}^+$ -independent and  $\text{Cl}^-$  - dependent manner with 1:1 exchange of extracellular cystine for intracellular glutamate [147] and is central to the production of glutathione in all cells [148] and astrocytes are no exception. Astrocytic regulation of system  $x_c^-$  has been studied extensively by the laboratory and previous studies have found that IL-1 $\beta$  enhances astrocytic system  $x_c^-$  expression and functional activity [67, 149]. Pertinent to this thesis, this increase has been shown to result in an enhancement of astrocytic GSH synthesis and its subsequent export [1]. Additionally, our laboratory demonstrated that IL-1 $\beta$  protects the astrocyte from oxidative stress through a GSH-dependent mechanism. Whether a similar effect is found in neurons or whether astrocyte GSH production can confer protection to neurons in co-culture is the subject of this thesis.

## 1.4. Specific Aim

**To study the neuroprotective potential of exogenous IL-1 $\beta$  against oxidative stress-mediated damage *in vitro*.**

The hypothesis that IL-1 $\beta$ -induced enhancement of astrocytic GSH protects neurons against oxidative stress was tested. Studies suggest that astrocytic enhancement of GSH is protective against oxidative damage in a cell-autonomous fashion. There is also strong evidence by others that astrocytes protect neurons (i.e., non-cell autonomously) from oxidative stress induced toxicity via GSH coupling. To test whether IL-1 $\beta$  can protect neurons either in a cell autonomous or non-cell autonomous manner, mixed cortical cell cultures containing neurons and astrocytes as well as near pure neuronal cultures were employed. Specifically, we aimed to decipher whether IL-1 $\beta$  can protect neurons in culture, much the way our laboratory has determined it protects astrocytes. Additionally, whether oxidative stress-induced neuronal injury is observed in chimeric cultures consisting of neurons derived from wild-type mice plated on top of astrocytes derived from IL-1R1 null animals (these animals lack the ability to signal through IL-1 $\beta$ ), which fail to show an increase in GSH synthesis and release following IL-1 $\beta$  exposure, was also assessed.



## Chapter 2. Materials and Methods

### 2.1 Experimental Animals

This study was conducted in accordance with the National Institute of Health guidelines for the use of experimental animals and has been approved by the Institutional Animal Care and Use Committee of Syracuse University. CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA) and were used in all experiments unless indicated otherwise. The *il1r1* null mutant animals (Strain: B6.129S7-IL1r1<sup>tm1lmx</sup>/J; JAX stock # 003245) [56] were purchased from JAX Labs (Bar Harbor, ME) and bred homozygously in parallel with animals from the background strain (JAX stock # 000664). Offspring from the C57BL/6J were used as wildtype controls (C57BL/6).

### 2.2. Cell Culture

Cell culture media and experimental buffer compositions were as follows: *Media stock* (MS): L-glutamine-free modified Eagle's medium (Earl's salt; MediaTech) supplemented with L-glutamine, glucose, and sodium bicarbonate to a final concentration of 2.0, 25.7, and 28.2 mM, respectively; *Astrocyte plating medium*: MS containing 10% fetal bovine serum (FBS; Hyclone) and 10% calf serum (CS; Hyclone), 10 ng/mL epidermal growth factor (Invitrogen), 50 IU penicillin, and 50 µg/mL streptomycin (Gibco); *Astrocyte maintenance medium*: MS containing 10% CS and 50 IU penicillin/ 50 µg/mL streptomycin. *Neuronal plating medium*: MS containing 5% bovine growth serum (BGS; Hyclone) and 5% CS (Hyclone),

50 IU penicillin, and 50 µg/mL streptomycin (Gibco); *Neuronal culture medium*: Neurobasal medium (NB) containing 1x B27 supplement (Invitrogen), 2 mM L-glutamine, 50 IU penicillin and 50 µg/mL streptomycin.

*Primary astrocytes* were cultured from pooled cortices of postnatal CD-1 pups (1-3 days; Charles River) as described in detail previously [150]. IL-1R1 wild-type and null mutant astrocytes were cultured from cerebral cortices of pups derived from IL-1R1 null homozygous breeding pairs. The remainder of brain tissue was used to confirm the genotype following dissection of the cerebral cortices, as described [<http://jaxmice.jax.org/strain/003245.html>].

In brief, following an aseptic dissection, cerebral cortices were placed in 0.025% trypsin diluted in Hank's balanced salt solution (Mediatek) and incubated for 15 min at 37°C. Dissociated cells were pelleted via centrifugation (3 min, 760 g), medium decanted and cells resuspended in astrocyte plating medium and plated at the equivalent of 2 hemispheres per 10 mL in 24-well plates (Falcon, Primaria). Upon confluence, monolayers of astrocytes were treated with 8 µM β-D-cytosine arabinofuranoside (AraC) once for 6-7 days to prevent microglial cell growth. Following this, astrocytes were fed every 7-8 days with astrocyte maintenance medium. Astrocytic cultures were used between 21-30 days *in vitro*. To remove any residual microglia before use in experiments, astrocyte monolayers were incubated with 50 mM of leucine methyl ester (LME) for 25-30 min one day prior to experimentation. Since C57BL/6J are more sensitive to LME, they were incubated with 50 mM LME for 20 minutes only.

*Primary near-pure neuronal cultures* were derived from the cortices of embryonic day 15 CD-1 mice, essentially as described above for astrocytes except that they were plated at a density of 1 million cells/mL in polyethyleneimine-coated plates in the neuronal plating medium. Four hours later, medium was exchanged and cells were placed in neuronal culture medium. After two days, neurons were treated with 1  $\mu$ M of AraC once for 2 days to prevent glial cell growth. Medium was partially replenished two days later, at 4 days *in vitro* by exchanging 250  $\mu$ L of medium with neuronal culture medium made using 1xB27 supplement devoid of antioxidants (B27 minus AO; Invitrogen) to remove existing antioxidants in the medium. Pure neuronal cultures were used at 6-7 days *in vitro*.

*Mixed cortical cell cultures* consisting primarily of neurons and astrocytes were prepared by plating dissociated cortical cells (0.75-0.8 million cells/mL) from embryonic day 15 CD-1 mouse fetuses on a confluent layer of astrocytes in neuronal plating medium. The medium was partially replaced after 5 and 9 days *in vitro* and treated with 8  $\mu$ M of AraC once at 7 days *in vitro*. Two days prior to experimentation, mixed cortical cell cultures were placed into media stock. Experiments were performed at 14 days *in vitro*. All cultures were kept at 37°C in a humidified 6% CO<sub>2</sub>-containing incubator.

### **2.3. IL-1 $\beta$ Treatment**

Cells were treated with various concentrations of recombinant murine IL-1 $\beta$  (R&D Systems) for various times in an incubation buffer of MS for astrocytes and mixed cultures and NB for neurons both supplemented with the vehicle, 0.1% fatty-acid

free BSA (Sigma). Cells were then returned to a humidified 37°C normoxic (21% O<sub>2</sub>) incubator containing 6% CO<sub>2</sub>.

#### **2.4. Measurement of GSH**

Total glutathione (GSH + GSSG) concentrations were measured both intracellularly ([GSH]<sub>i</sub>) and extracellularly ([GSH]<sub>e</sub>) using the GSH-Glo glutathione assay (Promega, Madison, WI) per manufacturer's instruction. Media was collected for analysis of [GSH]<sub>e</sub> and GSH Glo reaction buffer was added to the cells for lysis and analysis of [GSH]<sub>i</sub>. Samples were diluted using the reagent to keep relative light units within the dynamic range of the standards. To determine total GSH (reduced) levels, oxidized glutathione disulfide (GSSG) within the samples was converted to reduced GSH with the reducing agent TCEP-HCl (final concentration = 1 mM; 10 min; 25°C; Thermo Scientific; Waltham, MA). Luciferase activity was quantified using a Synergy 2 microplate reader (BioTek, Winooski, VT). Total intracellular or extracellular GSH were normalized to standards prepared in GSH-Glo reagent and MS containing L-glutamine and 0.1% fatty acid free BSA, respectively. Intracellular GSH was normalized to cellular protein quantified using BCA Assay Kit (Thermo Scientific) as per the manufacturer's instructions. GSH standards were linear over the range of 0 to 5 μM.

## 2.5. Drug Exposure

**Tert-butyl Hydroperoxide (t-BOOH) Treatment:** t-BOOH is a direct-acting organic peroxide used to induce oxidative stress, the clearance of radicals generated by which is GSH-dependent. A stock solution of t-BOOH (1.5 M in H<sub>2</sub>O; Acros Organics) was made and stored at 4°C. t-BOOH was added to the cultures (final concentration: 0.1–4 mM) in treatment media (MS for astrocytes and mixed cultures and NB for neurons). Experiments were terminated 2.5 to 3.5 h later and tissue culture supernatant was removed for the measurement of lactate dehydrogenase (LDH) activity as an assessment of cell death. Following the harvest of samples for LDH assay, MTT assay was carried out by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to assess for cell survival. Minimum incubation for MTT assay: 2 h.

**MK-571:** MK-571 is a potent inhibitor of the Mrp1 transporter, a transporter used by astrocytes to export GSH. A stock solution of MK-571 (25 mM in H<sub>2</sub>O; Enzo Life Sciences) was made and stored at -20°C. MK-571 was added to the cultures (final concentration: 50-70 µM) in treatment media (MS for astrocytes and mixed cultures). After 1.5 h of incubation, wells were treated with IL-1β for 24 h followed by challenge with t-BOOH as described in each figure legend. Experiments were terminated 2.5 to 3.5 h later by addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to assess for cell survival.

**Glutathione Ethyl Ester (GEE) addition:** GEE is a membrane permeable derivative of GSH that is used to supplement GSH supply within cells. A 0.5 M

stock of GEE (Sigma) was made freshly for each experiment in NB. GEE was added to neuronal cultures at final concentration of 0.5 to 3 mM.

## **2.6. Measurement of Cell Death and Viability**

**Cell Death:** Cell death was quantitatively determined by spectrophotometric measurement of lactate dehydrogenase (LDH) as described previously [151]. In short, LDH activity was determined by measuring the pyruvate-dependent rate of oxidation of NADH. To do so, 40  $\mu$ L tissue culture supernatant were added to potassium phosphate buffer (100 mM, pH 7.4, 150  $\mu$ L). Then, NADH (0.4 mM, 125  $\mu$ L), followed by pyruvate (23 mM, 50  $\mu$ L) was added for a total reaction volume of 375  $\mu$ L. Immediately following the addition of pyruvate, the loss of absorbance at 340 nm - an index of NADH oxidation - was recorded every 34 s for a period of 4 min using a microplate reader SpectraMax M2 (Molecular Devices). A standard curve was constructed using Level II Control Serum (Pointe Scientific Inc.), from which LDH concentration in the experimental medium was calculated via linear regression. Astrocyte and mixed culture injury was quantified as a percentage of total astrocytic LDH activity (defined as 100%) determined by exposure of parallel cultures to 20  $\mu$ M Calphostin C for 20-24 h [152].

**Cell Viability:** Cell viability was quantified via colorimetric analysis of MTT (Sigma) reduction as previously described [153]. Following treatment, MTT was added to the cultures (final concentration = 300  $\mu$ g/mL) for at least 3 h at 37°C, after which the solution was carefully aspirated, and the resulting crystals solubilized in acidified isopropanol (90% isopropanol; 10% 1 N HCl; 400  $\mu$ L/well).

200  $\mu$ L was transferred to a 96-well plate and absorbance at 540 nm was measured against a 690 nm background subtraction (SpectraMax M2, Molecular Devices). Percent viable astrocytes was quantified by normalization of experimental MTT absorbance values to values obtained from untreated control cells (i.e., highest absorbance = 100%) as well as cells treated with 1.5 mM t-BOOH, 20  $\mu$ M Calphostin C or 300  $\mu$ M NMDA, which result in complete loss of viability (defined as 0%).

## **2.7. Propidium Iodide Staining**

In some mixed culture experiments, cell death was additionally assessed via propidium iodide (PI; Molecular Probes) staining [154]. Live cells exclude PI. In short, PI (10 $\mu$ g/mL) was added to culture wells for 10 min, after which PI was removed by gentle washing with PBS (3 x 750  $\mu$ L) and imaged. Fluorescent photos were acquired by a DP73 digital color camera (Digital Video Camera Co.) mounted on an Olympus IX50 inverted microscope outfitted with epifluorescence controlled by CellSens Standard (Olympus) software. Brightness and contrast were standardized for each picture.

## **2.8. Statistical Analysis**

All statistical analyses were performed using GraphPad Prism Version 6.0 as described. Percentage data were transformed (arcsin square root) before analysis because it is non-normally distributed. In all experiments, data are expressed as the mean + SEM. Significance was assessed at  $p < 0.05$ .

## Chapter 3. Results

The goal of the following experiments was to test the protective potential of IL-1 $\beta$  against t-BOOH injury employing astrocytes, neurons and co-cultures of the two. The results have been accordingly divided into 3.1 for astrocytes, 3.2 for neurons and 3.3 for mixed culture and 3.4 for neurons + GEE.

### 3.1. Astrocytes

In confirmation of previous findings in the laboratory [1]: 1) treatment of enriched cortical astrocytes with IL-1 $\beta$  (5 ng/ mL) for 48 h resulted in an increase in the extracellular GSH that accumulated in the supernatant, while the intracellular levels remained unchanged (Figure 3); 2) t-BOOH toxicity to astrocytes was abolished by IL-1 $\beta$  pretreatment (Figure 4); 3) astrocytic cultures derived from *il1r1* null mutant mice, that is mice lacking the IL-1 $\beta$  signaling receptor, show no increase in extracellular GSH following stimulation with IL-1 $\beta$  (Figure 5A), nor do these cells enjoy any protection when treated with t-BOOH, as compared with astrocytes derived from wild-type control mice (Figure 5B).

Interestingly, the basal extracellular GSH levels as well as the IL-1 $\beta$ -mediated enhancement were concentration-dependently blocked by concomitant treatment of astrocytes with MK-571 (50–70  $\mu$ M), suggesting that release occurred via Mrp1, as has been reported previously [155] (Figure 6A). Despite this, the toxicity of t-BOOH and the protection afforded by IL-1 $\beta$  pre-treatment was not appreciably affected by MK-571 (Figure 6B).



### 3.2. Neurons

We next set out to determine whether IL-1 $\beta$  pre-treatment could affect neuronal GSH levels. Unlike in astrocytes, treatment of enriched cortical neurons with 5 ng/ mL of IL-1 $\beta$  for 48 h did not cause any change in the levels of intracellular or extracellular GSH (Figure 7), begging the question as to whether it would protect neurons in isolation against an oxidant stressor. In order to test this, we first needed to find a t-BOOH concentration that would facilitate neuronal injury in a similar exposure time frame as astrocytes. As demonstrated in Figure 8, lower concentrations of t-BOOH effectively resulted in neuronal cell death, but they required longer exposure. Concentrations of t-BOOH between 1-4 mM resulted in  $57.5\% \pm 3.03$  cell death in a time frame similar to that used in astrocyte experiments (3.5 h) and as such these concentrations were chosen for subsequent experiments with neurons. Exposure of neurons to increasing concentrations of t-BOOH (1, 2 and 4 mM) resulted a concentration-dependent toxicity that was unabated by treatment with IL-1 $\beta$  (5 ng/ mL; 48 h) (Figure 9). To ensure that the lack of protection was not a result of sub-optimal IL-1 $\beta$  concentrations, neurons were treated with increasing concentrations of IL-1 $\beta$  (5-20 ng/ mL; 48 h) and then challenged with 2 mM t-BOOH. At every concentration tested, IL-1 $\beta$  failed to rescue neurons from t-BOOH toxicity (Figure 10). Given the fact that IL-1 $\beta$  treatment also failed to raise the levels of neuronal GSH level, these results might not be unexpected.

### 3.3. Mixed culture

Astrocytes and neurons have been shown to function together and have beneficial interactions in the CNS. Since the data demonstrated that astrocytes responded to IL-1 $\beta$  by releasing GSH, we next investigated if this astrocytic response could be neuroprotective in a co-culture of astrocytes and neurons. As both neurons and astrocytes have functional IL-1R1 receptors, we chose a higher concentration of IL-1 $\beta$  (10 ng/ mL) for treating the cells to ensure that astrocytes signaling would be optimal (S Hewett Lab, unpublished observations). Treatment of mixed culture with IL-1 $\beta$  (10 ng/ mL; 48 h) resulted in a significant increase in the levels of extracellular GSH (Figure 11). Additionally, cells in mixed culture receiving the IL-1 $\beta$  pretreatment were protected against t-BOOH toxicity. Specifically, t-BOOH exposure reduced the cell viability by approximately 55%  $\pm$  4.6 for 1.5 mM t-BOOH and 60%  $\pm$  4.6 for 2 mM t-BOOH, while cell viability in the IL-1 $\beta$ -treated group was only reduced by 20%  $\pm$  4.4 for 1.5 mM t-BOOH and 32%  $\pm$  6.3 for 2 mM t-BOOH (Figure 12). Using propidium idodide (PI) to stain dead and dying cells, we found that both neurons and astrocytes were vulnerable to t-BOOH toxicity and that both cell types were protected by IL-1 $\beta$  pretreatment (Figure 13).

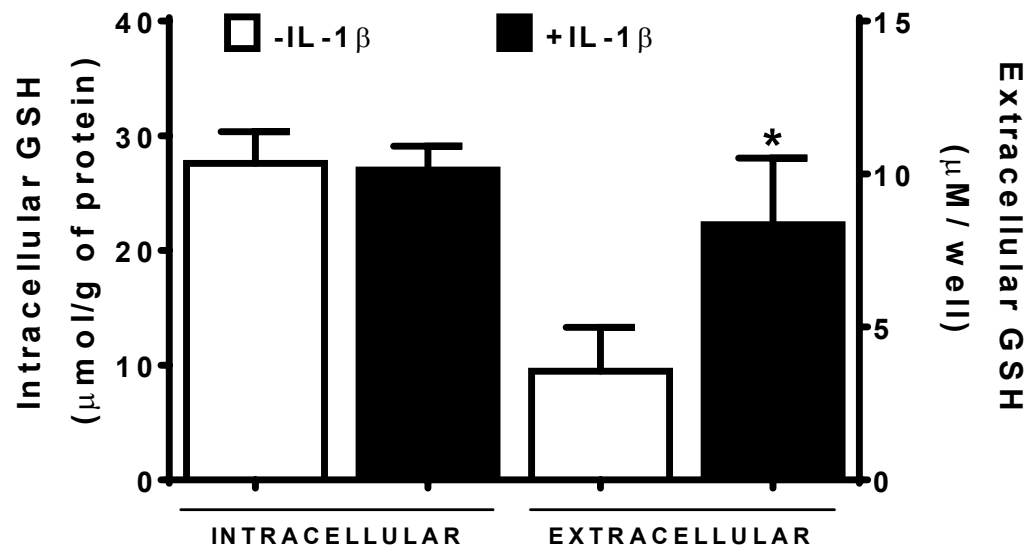
To investigate whether the protective effect of IL-1 $\beta$  was dependent on astrocyte signaling, we generated chimeric cultures in which either *il1r1*  $+/+$  or  $-/-$  astrocytes were co-cultured with wild-type neurons. Cultures containing *il1r1*  $-/-$  did not respond to IL-1 $\beta$  treatment (10 ng/ mL, 48 h) with an increase in GSH

synthesis and release as compared to those containing *il1r1* +/+ (Figure 14). Additionally, chimeric cultures containing *il1r1* -/- were not protected against t-BOOH toxicity (1.5 mM t-BOOH; 2 h) by prior IL-1 $\beta$  treatment (10 ng/ mL, 48 h) whereas those containing *il1r1* +/+ astrocytes were rescued (Figure 15).

Finally to determine whether extracellular GSH release was necessary for the protective effect of IL-1 $\beta$  in the mixed culture system, release of GSH was abrogated by treatment with the Mrp1 blocker, MK-571 (50–70  $\mu$ M) (Figure 16A). Interestingly, the protective effect of IL-1 $\beta$  was significantly attenuated when GSH release was reduced (Figure 16B). These findings point towards the antioxidant aspect of the intricate cross-talk between astrocytes and neurons in the brain. Unfortunately, we did not assess the potential cell-type specificity of the response.

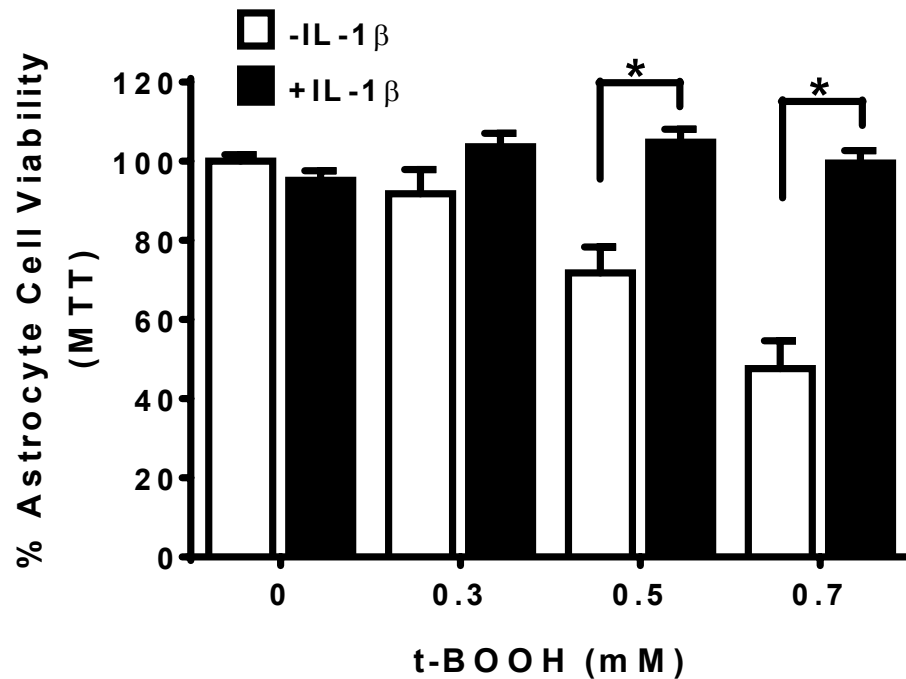
### **3.4. Neurons + GEE**

To determine whether supplementing neurons in near pure culture with external glutathione could substitute for the presence of astrocytes, we provided neurons in culture with glutathione ethyl ester (GEE, 0.5–3 mM) (Figure 17A), then challenged them with 2 mM t-BOOH for 3 h (Figure 17B). The intracellular GSH increased significantly upon treatment with 3 mM GEE; however, GEE treatment did not rescue neurons from t-BOOH toxicity and in fact it was toxic on its own (Figure 17B). Thus, we can draw no conclusions regarding the role of GSH supplementation using this paradigm.

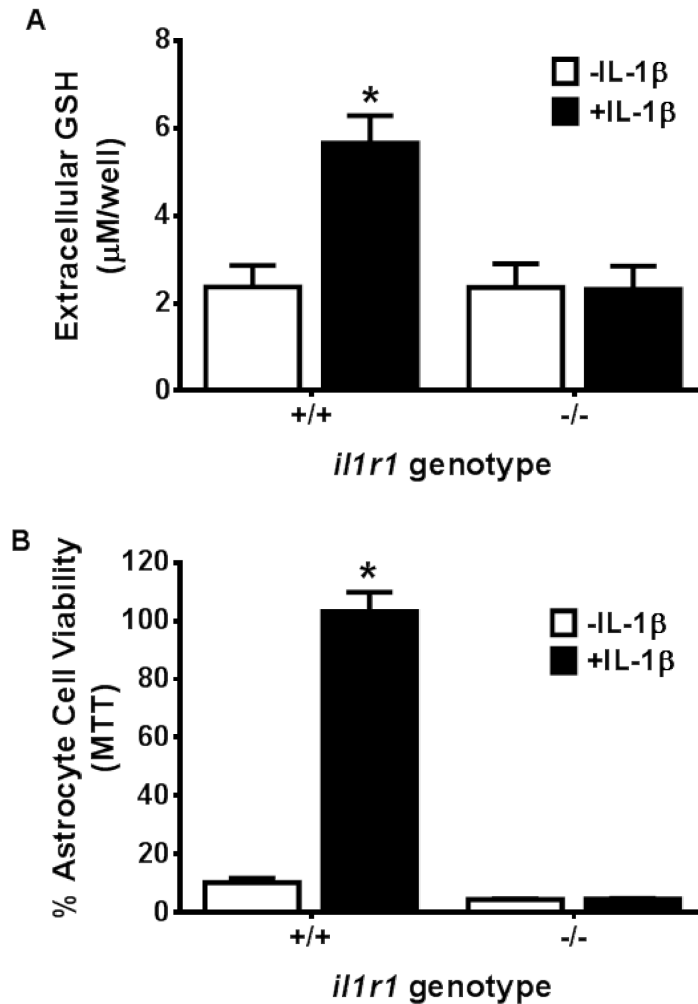


**Figure 3: IL-1 $\beta$  pre-treatment increases the GSH status of astrocytes.**

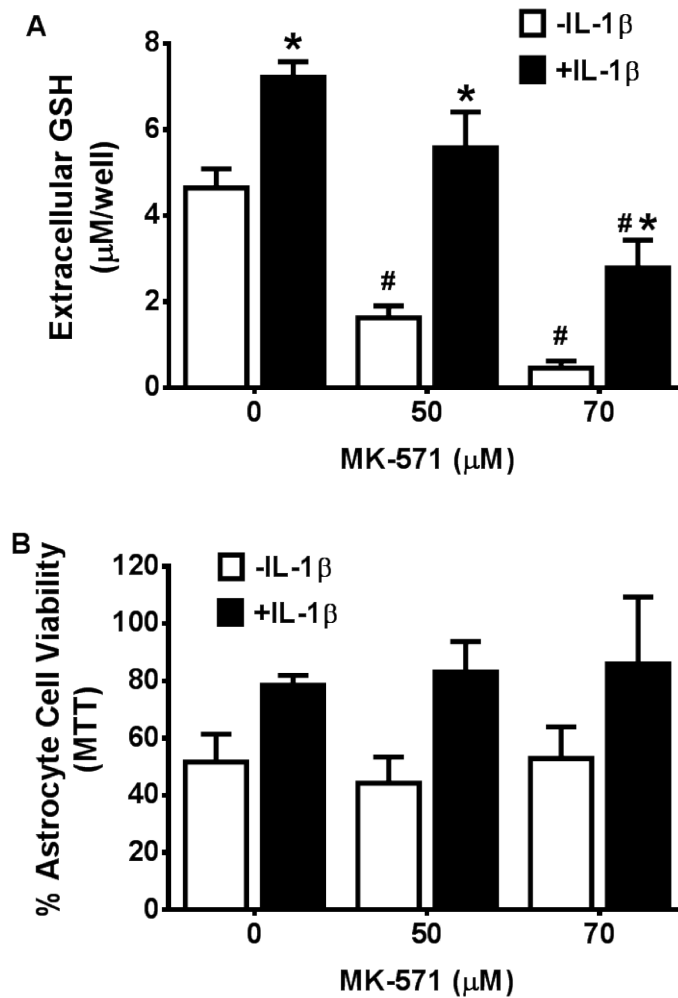
Astrocytes cultured from CD-1 mice (400  $\mu$ L/well,  $0.16 \pm 0.007$  mg protein/well) were washed twice with treatment media and incubated with IL-1 $\beta$  (5 ng/mL) or vehicle for 48 h, after which total intracellular ( $n = 6$  from two separate dissections) and extracellular ( $n = 12$  from 4 separate dissections) GSH levels were measured. Data are expressed as mean + SEM. An asterisk (\*) denotes a significant between-group difference (comparisons between vehicle and IL-1 $\beta$  treated cultures) as assessed by Mann-Whitney U test ( $p = 0.0083$ ).



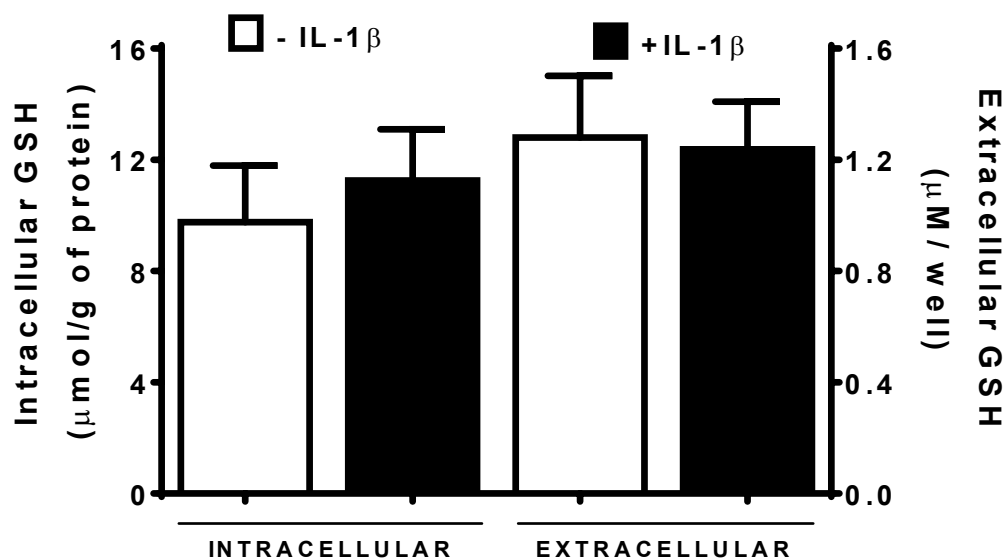
**Figure 4: IL-1 $\beta$  pre-treatment protects pure astrocytes against t-BOOH-mediated cell death.** Astrocytes cultured from CD-1 mice were washed twice with treatment media and incubated with IL-1 $\beta$  (5 ng/mL) or vehicle for 48 h after which they were treated with the t-BOOH concentrations indicated for 3.25 h. Cell viability was determined via the measurement of mitochondrial succinate dehydrogenase using the MTT assay. Data are expressed as % astrocyte viability (mean + SEM) normalized to MTT values found in the untreated group; n = 9-10 from two separate dissections. An asterisk (\*) denotes significant between-group differences (+ and - IL-1 $\beta$  at each t-BOOH concentration) as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons ( $p \leq 0.0002$ ).



**Figure 5: Increase in extracellular GSH and protection against t-BOOH-mediated cell death in astrocytes is purely due to IL-1 $\beta$  signaling through IL-1R1.** (A) Astrocytes ( $n = 12$  from two separate dissections) from *il1r1* +/+ or -/- mice were washed twice with treatment media and incubated with IL-1 $\beta$  (5 ng/mL) or vehicle for 48 h after which total supernatant GSH levels were measured. (B) Astrocytes from the same dissections ( $n = 13-15$ ) were treated with 0.7 mM t-BOOH for 2.25 h, after which experiments were terminated by addition of MTT to assess for cell survival. Data are expressed as mean + SEM. For B, viability data are expressed as % astrocyte viability normalized to MTT values found in the untreated group (= 100%). An asterisk (\*) denotes significant between-group differences (+ and - IL-1 $\beta$  in the same genotype) as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons ( $p = 0.0007$  for A and  $p < 0.0001$  for B).

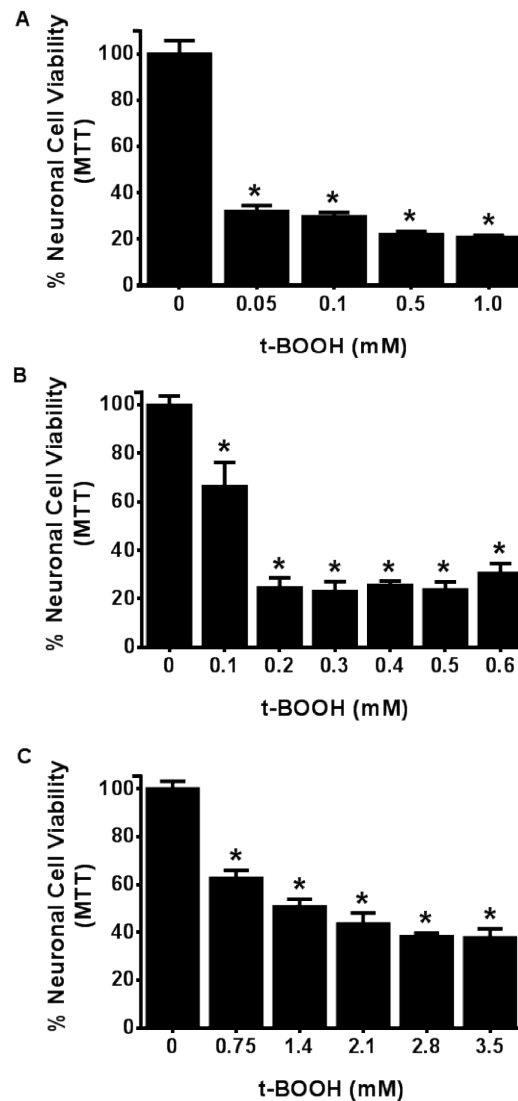


**Figure 6: Attenuation of astrocytic GSH extrusion does not affect the cellular viability upon t-BOOH challenge.** Astrocyte cultures derived from CD-1 mice were washed twice with treatment media and incubated with different concentrations of MK-571 as indicated for 1.5 h, after which the wells were treated with IL-1 $\beta$  (5 ng/mL) or vehicle for an additional 24 h. Thereafter, total supernatant GSH levels were measured (A) Subsequently, wells were challenged with 1.5 mM t-BOOH for 2.45 h after which cell viability was determined via the MTT assay (n = 8 from four separate dissections). (B). Data are expressed as mean + SEM. For B, viability data are expressed as % astrocyte viability normalized to MTT values found in the untreated group (= 100%) (n = 6-8 from four separate dissections). An asterisk (\*) denotes significant between-group difference (+ and – IL-1 $\beta$  with or without MK-571) and a pound (#) denotes significant within-group difference (+ and – IL-1 $\beta$  treated) as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. ( $p = \leq 0.035$  for \* and  $p \leq 0.0054$  for #).

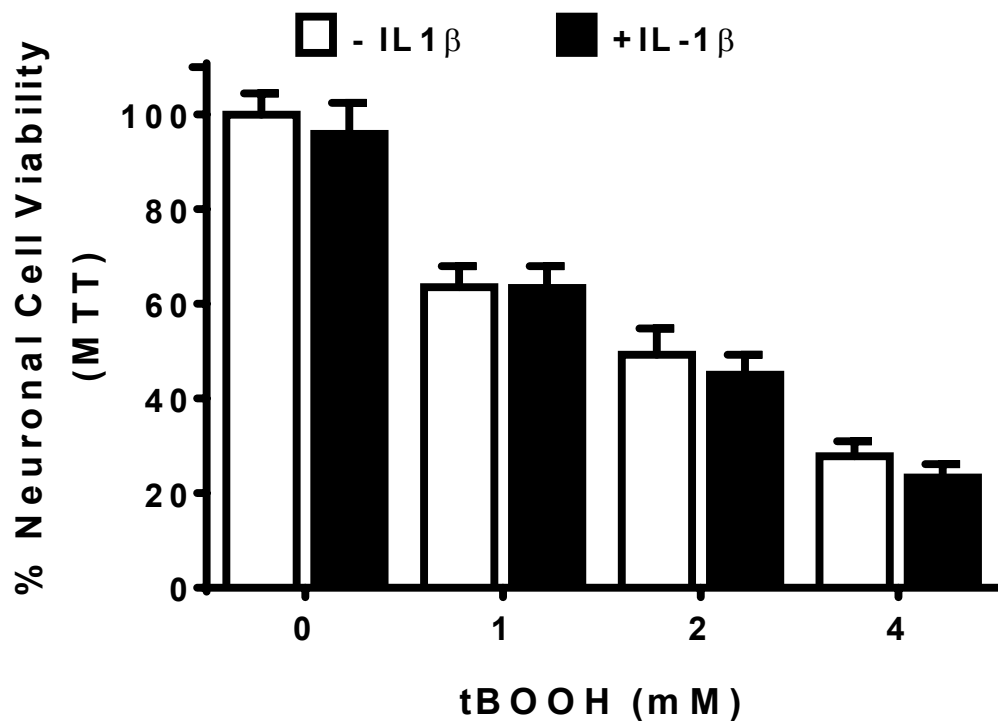


**Figure 7: IL-1 $\beta$  pre-treatment does not change the GSH status of neurons.** Neurons cultured from CD-1 mice were washed twice with treatment media and incubated with IL-1 $\beta$  (5 ng/mL) or vehicle for 48 h, after which total intracellular and supernatant GSH levels were measured (well volume = 400  $\mu$ L,  $0.36 \pm 0.024$  mg protein/ well). Data are expressed as mean + SEM. No significant difference was observed between groups (vehicle vs. IL-1 $\beta$  treated cultures) as assessed by two-way ANOVA ( $n = 9$  from three separate dissections).

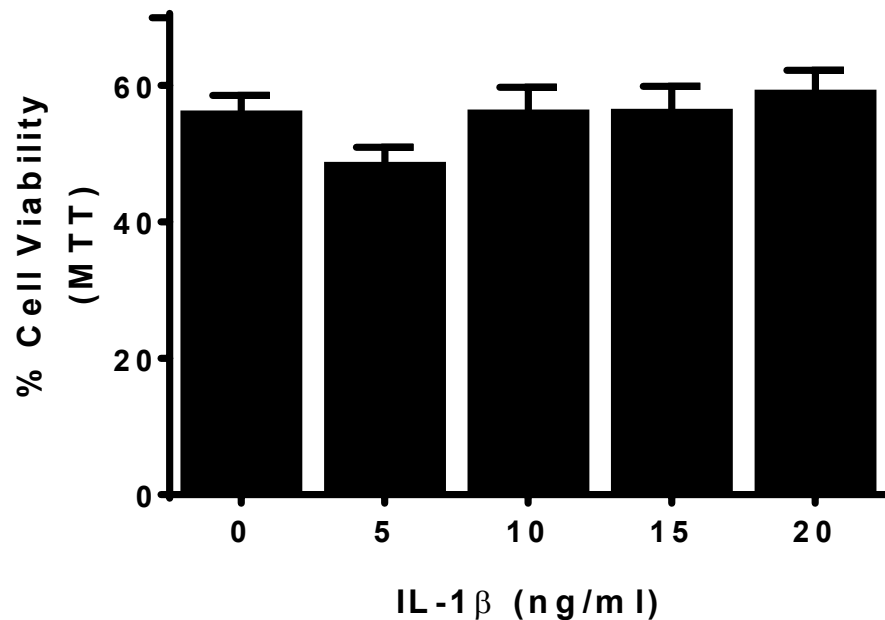




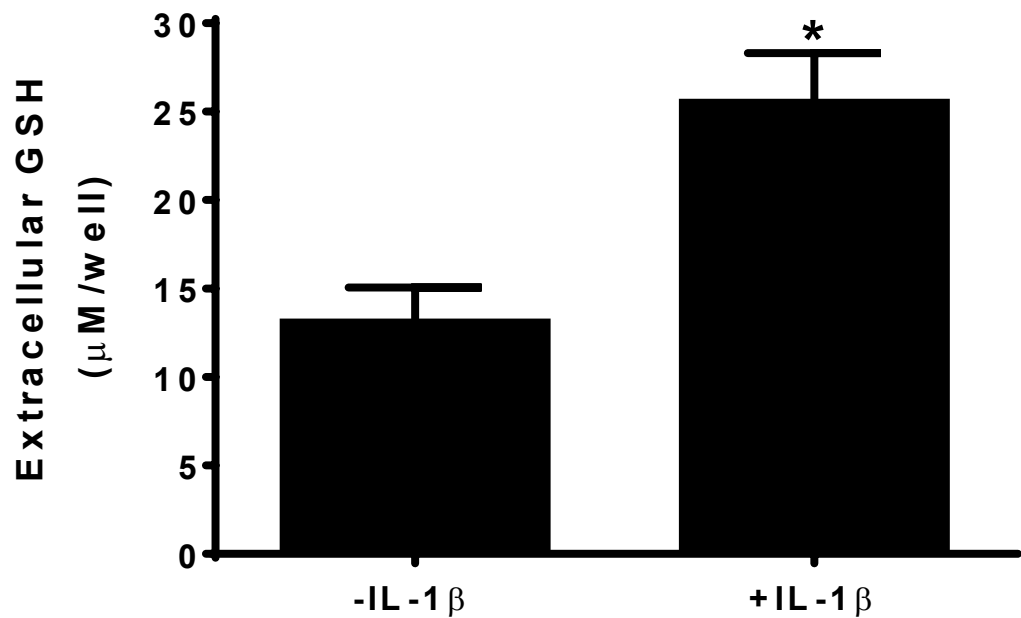
**Figure 8: t-BOOH causes cell death in a concentration-dependent manner in neurons.** Neurons cultured from CD-1 mice were washed twice with treatment media and incubated for (A) 6.5 h (n = 8 from two independent experiments) (B) 7 h (n = 8-9 from three independent experiments) or (C) 3.5 h (n = 4-8 from two independent experiments) with the t-BOOH concentrations as indicated. Neuronal viability was determined by the MTT assay. Data are expressed as % mean neuronal viability + SEM normalized to non-treated group (= 100%). Asterisks (\*) denote significant differences between untreated cultures (0 mM) and t-BOOH treated cultures as assessed by one-way ANOVA followed by Dunnett's test for multiple comparisons.  $p \leq 0.0017$  for A,  $p < 0.0001$  for B and  $p < 0.0001$  for C.



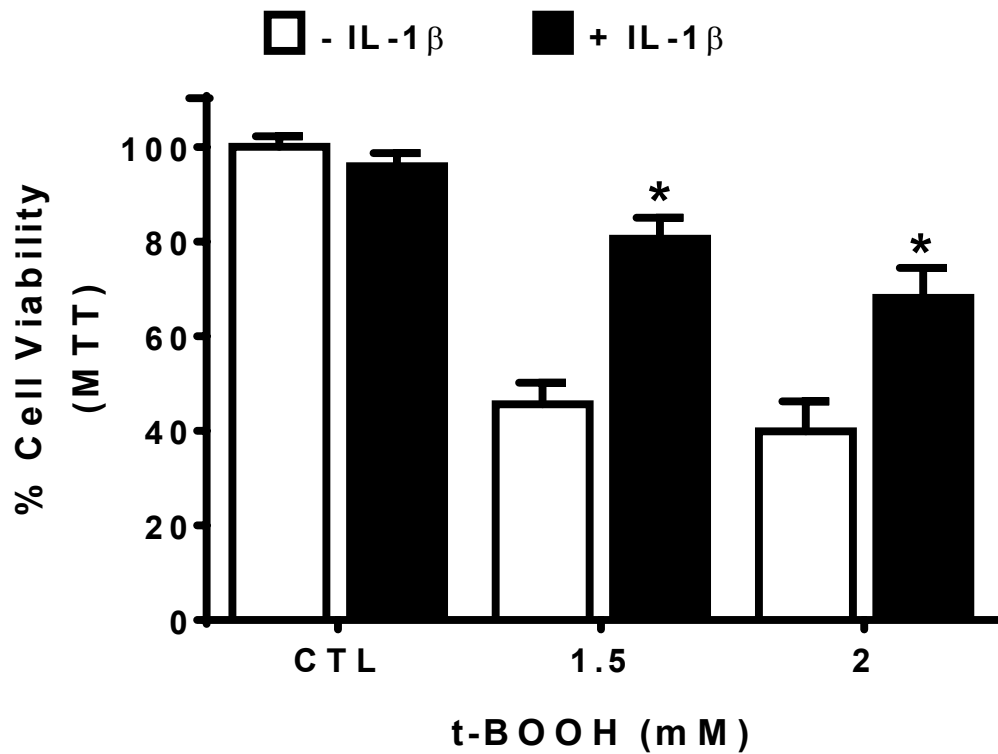
**Figure 9: IL-1 $\beta$  pre-treatment does not protect neurons against t-BOOH-toxicity.** Neurons cultured from CD-1 mice were washed twice with treatment media and incubated with IL-1 $\beta$  (5 ng/mL) or vehicle for 48 h. Next cells were treated with the t-BOOH concentrations indicated (1.25 to 4.5 h) and cell viability was determined using the MTT Assay. Data are expressed as % neuronal cell viability (mean + SEM) normalized to MTT values of the untreated group (= 100%). No significant between-group differences (+ and - IL-1 $\beta$  at each t-BOOH concentration) were observed as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons (n = 6-9 from two separate dissections).



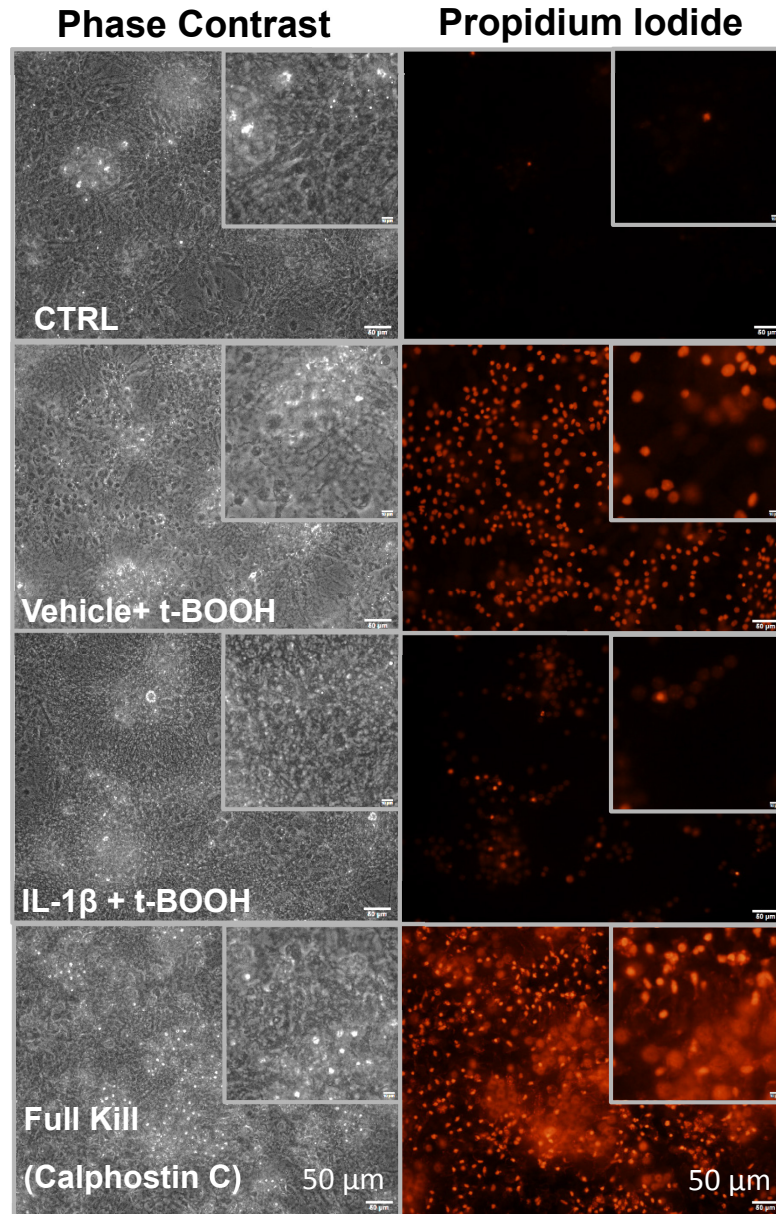
**Figure 10: Increasing the concentrations of IL-1 $\beta$  pre-treatment does not protect pure neurons against t-BOOH-mediated cell death.** Neurons cultured from CD-1 mice were washed twice with treatment media and incubated with different concentrations of IL-1 $\beta$  or vehicle as indicated in the figure. 48 h later, cultures were treated with 2 mM t-BOOH for 3.5-4 h after which the experiment was terminated by addition of MTT to assess for cell survival. Because there was no significant difference in cell viability between each respective control (i.e., vehicle-treated group for each IL-1 $\beta$  concentration), values were pooled and are represented as a single group for ease of presentation. Data are expressed as % mean neuron viability + SEM, normalized to MTT values of the untreated control cultures (= 100%). No significant between-group difference was observed as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. (n = 7-8 from two separate dissections).



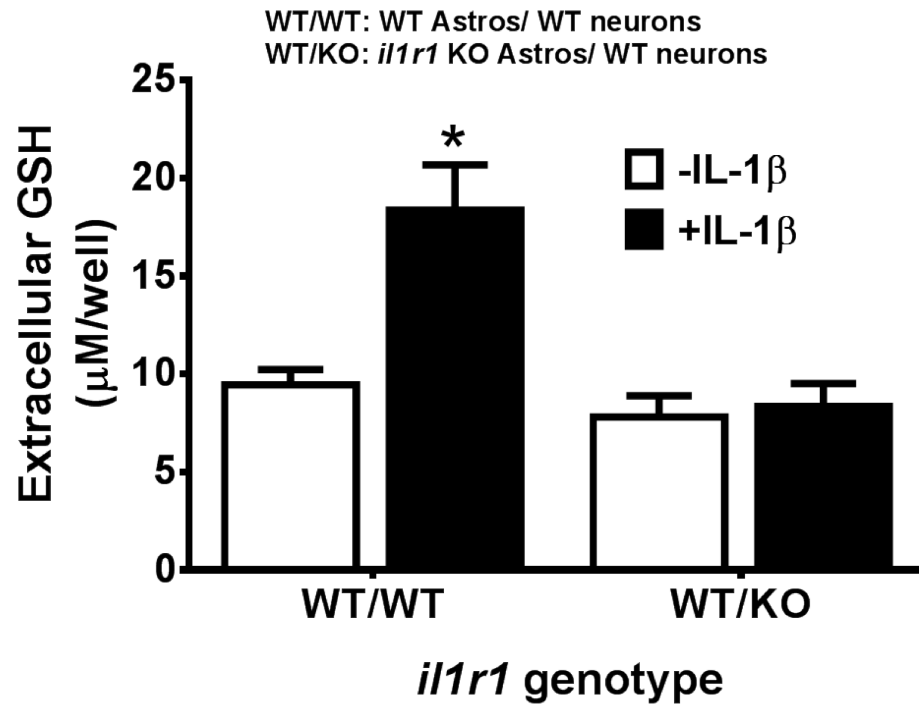
**Figure 11: IL-1 $\beta$  pre-treatment increases the GSH release from mixed cultures.** Mixed cortical cultures from CD-1 mice were washed twice with treatment media and incubated with IL-1 $\beta$  (10 ng/mL) or vehicle for 48 h after which total supernatant GSH levels were measured. Data are expressed as mean  $\mu\text{M}/\text{well}$  + SEM. An asterisk (\*) denotes significant difference as assessed by Student's t-test ( $p = 0.0006$ ) ( $n = 22\text{-}24$  from five separate dissections).



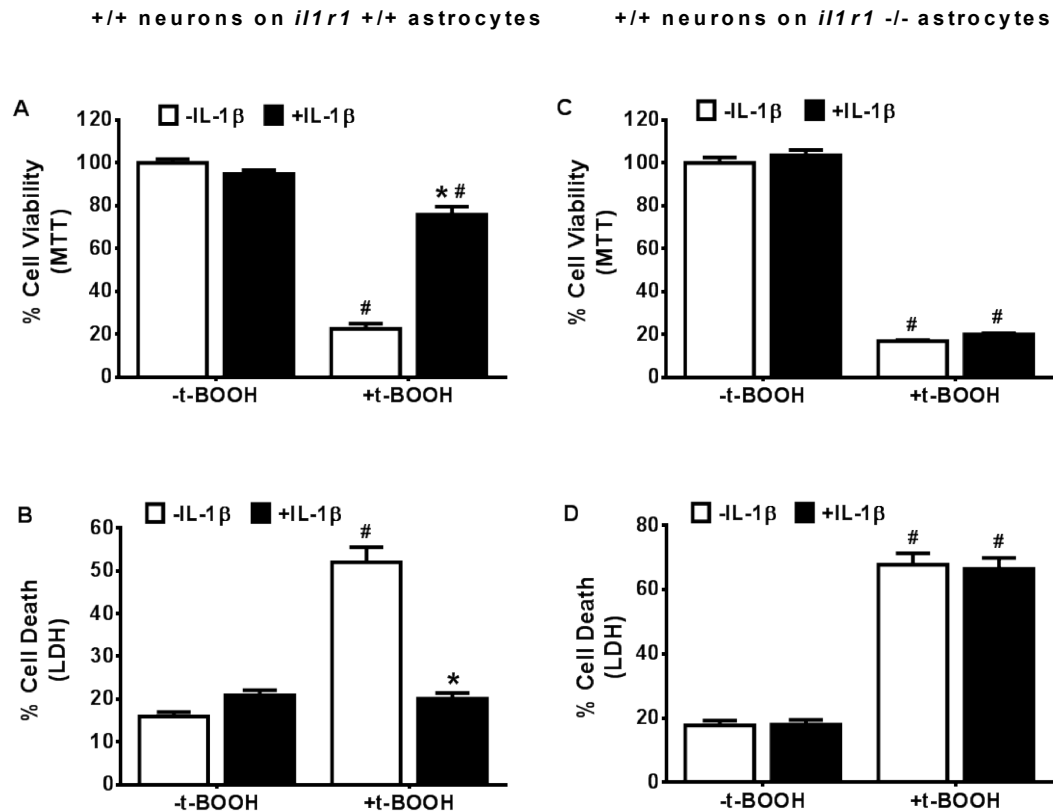
**Figure 12: IL-1 $\beta$  pre-treatment protects mixed cultures against t-BOOH toxicity.** Mixed cortical cell cultures from CD-1 mice were washed twice with treatment media and incubated with IL-1 $\beta$  (10 ng/mL) or vehicle for 48 h. Next, cultures were treated with t-BOOH at concentrations indicated for 2.75-3.25 h. The experiment was then terminated by addition of MTT to assess for cell survival. Data are expressed as % mean cell survival + SEM normalized to MTT values of the non-treated control cultures (= 100%). An asterisk (\*) denotes a significant between-group difference (+ and - IL-1 $\beta$  for a given t-BOOH concentration) as determined via two-way ANOVA followed by Bonferroni's test for multiple comparisons (n = 7-16 from three separate dissections;  $p \leq 0.0013$ ).



**Figure 13: IL-1 $\beta$  pre-treatment of mixed culture protects both astrocytes and neurons.** Mixed cultures consisting of neurons and astrocytes from CD-1 mice were washed twice with treatment media and incubated with IL-1 $\beta$  (10 ng/mL) or vehicle for 48 h after which they were treated with 1.5 mM t-BOOH for 2.75 h. Cell death was determined qualitatively by fluorescence microscopy after terminating the toxicity experiment via washing thrice with PBS followed by treatment with propidium iodide (10  $\mu$ g/mL). Representative photos depict phase contrast (left panel) and propidium iodide staining (right panel).

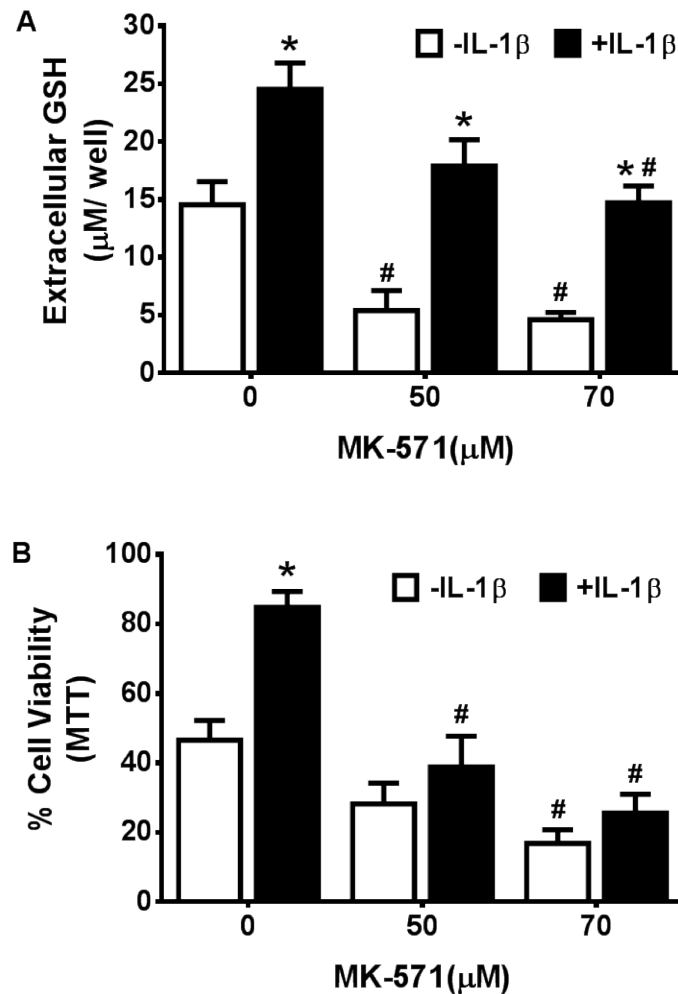


**Figure 14: Increase in extracellular GSH in chimeric culture is astrocyte IL-1R1-dependent.** Wild-type neurons were plated on astrocytes from *il1r1* +/+ or -/- mice. Mixed cultures were washed twice with treatment media and incubated with IL-1β (10 ng/mL) or vehicle for 48 h after which total supernatant GSH levels were measured. Data are expressed as mean μM GSH/well + SEM. An asterisk (\*) denotes significant between-group difference (+ and – IL-1β within the same combination of chimeric culture) as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. (n =8 from two separate dissections; *p* = 0.001).

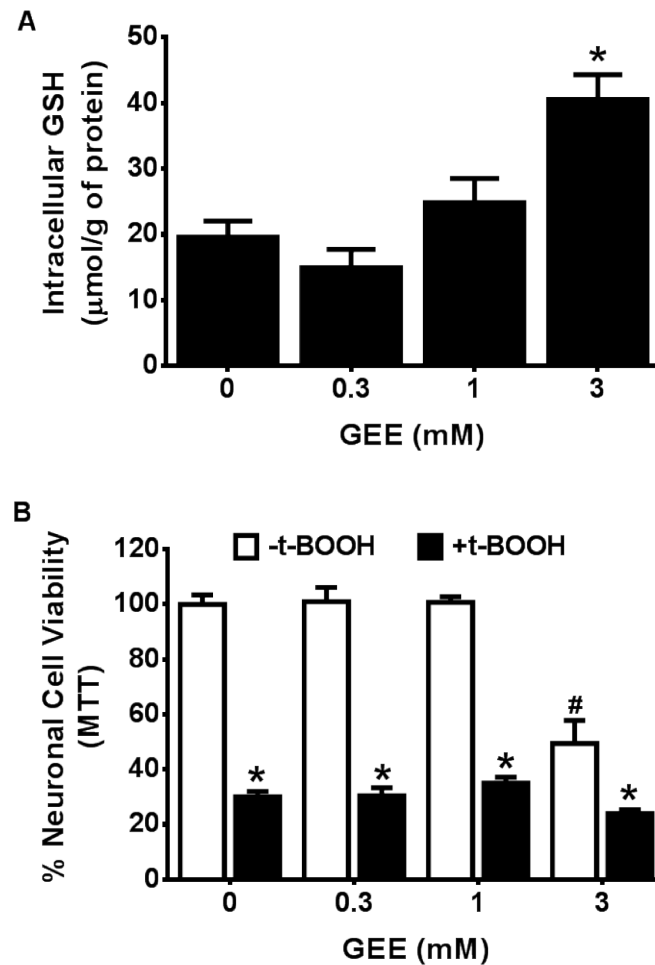


**Figure 15: IL-1 $\beta$  pre-treatment protects neurons plated on *il1r1* +/+ but not *il1r1* -/- astrocytes in chimeric mixed culture against t-BOOH-mediated cell death.** Astrocytes from either *il1r1* +/+ or -/- mice, plated with neurons from CD-1 wild-type mice were washed twice with treatment media and incubated with IL-1 $\beta$  (10 ng/mL) or vehicle for 48 h after which they were treated with 1.5 mM t-BOOH for 1.25-1.5 h. This was followed by determination of toxicity by obtaining supernatant from the wells and measurement of LDH release for cell death (B and D) as well as addition of MTT to assess for cell survival (A and C). MTT incubation = 2 h. For MTT, data are expressed as mean cell survival + SEM normalized to non-treated control cultures (= 100%). For LDH, data are expressed as mean cell death + SEM normalized to the Calphostin C-treated group (= 100%). An asterisk (\*) denotes significant between-group difference (+ and - IL-1 $\beta$  with or without t-BOOH) and a pound (#) denotes significant within-group difference (+ and - IL-1 $\beta$  treated) as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. [n=24-26 (A and B), n=18-22 (C and D);  $p < 0.0001$ ].





**Figure 16: Attenuation of astrocytic GSH extrusion decreases cellular viability in mixed culture upon t-BOOH treatment.** Mixed cultures derived from CD-1 mice (n = 8 from three separate dissections) were washed twice with treatment media and incubated with different concentrations of MK-571 as indicated for 2 h, after which the wells were treated with IL-1 $\beta$  (10 ng/mL) or vehicle for an additional 24 h. (A) Total supernatant GSH levels expressed as mean  $\mu$ M/well + SEM (B) Mean % cell survival normalized to MTT values of the non-treated control cultures (= 100%) following challenge with 1.5 mM t-BOOH for 2.45 h. An asterisk (\*) denotes significant between-group difference (+ and – IL-1 $\beta$  with or without MK-571) and a pound (#) denotes significant within-group difference (+ and – IL-1 $\beta$  treated) as assessed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. Significance was assessed at  $p < 0.05$ .



**Figure 17: Supplementation of pure neurons with GSH-Ethyl Ester (GEE) does not affect the cell viability upon t-BOOH treatment.** Neurons from CD-1 mice were washed twice with treatment media and incubated with increasing concentrations of Glutathione-Ethyl ester (GEE). (A) Total intracellular GSH levels expressed as mean  $\mu\text{mol}$  GSH per gram of protein ( $n = 4-8$ ). An asterisk (\*) denotes values different from control (0 mM GEE) as determined by one-way ANOVA followed by Dunnett's t-test ( $p = 0.0013$ ) (B). Mean % cell survival normalized to MTT values of the non-treated control cultures (= 100%) following exposure to 2 mM t-BOOH for 3 h ( $n = 3-6$ ). An asterisk (\*) denotes significant between-group difference (+ and -t-BOOH for a given concentration of GEE) and a pound (#) denotes significant within-group difference (+ and - t-BOOH) as determined by two-way ANOVA followed by Bonferroni's test for multiple comparisons. ( $p < 0.0001$ ).

## Chapter 4. Discussion

Oxidative stress and neuroinflammation have been found to coexist in a number of debilitating diseases [156-158]. Models that combined oxidative stress and endogenous neuroinflammation were used in the study. We specifically aimed to determine whether the neuroinflammatory cytokine IL-1 $\beta$  is inherently protective against an oxidant stress effected using t-BOOH, and if so, whether GSH was the predominant mediator of this effect.

The results of the work are as follows:

- 1) t-BOOH causes a concentration-dependent cell death in both neurons and astrocytes.
- 2) IL-1 $\beta$  enhances GSH and confers protection against t-BOOH to astrocytes cultured alone but not to neurons in isolation.
- 3) IL-1 $\beta$  enhances GSH in mixed cortical cell culture and confers protection against t-BOOH to both neurons and astrocytes in co-culture.
- 4) Astrocytic signaling is required to observe IL-1 $\beta$ -mediated GSH increase and neuroprotection.
- 5) Blocking the astrocytic release of GSH abrogates protection conferred to mixed cultures.
- 6) Addition of the GSH analogue, GEE, at concentrations that are not toxic to the cells, does not elevate neuronal GSH levels, nor does it provide the

neurons any protection from t-BOOH toxicity. Hence, we can draw no conclusions regarding the role of GSH supplementation using this paradigm.

Oxidative stress has been implicated as a contributory factor to neuronal damage in many neurological diseases and pathologies [159, 160]. These same disorders/diseases also show a component of a neuroinflammatory response [161-163]. Inflammatory response can be causative or reparative depending on the interplay of various cellular pathways. IL-1 $\beta$  has been shown to be a 'master regulator' of neuroinflammation [164]. IL-1 $\beta$  has been at the crossroads of repair and injury [6, 11, 63, 165]. Studies from our laboratory have shown both protective and detrimental effects of IL-1 $\beta$ . It was shown that IL-1 $\beta$  potentiates hypoxic neuronal injury in *in vitro* and *in vivo* models of cerebral ischemia. The *il1r1* null mice showed smaller infarcts and less neurological deficits. *In vitro*, IL-1 $\beta$  exacerbated hypoxia-induced neuronal death [67]. IL-1 $\beta$ -mediated regulation of astrocytic system  $x_c^-$  is responsible for its excitotoxic effects under hypoxic conditions [149]. In contrast, IL-1 $\beta$  was glioprotective against direct oxidative injury, in a glutathione-dependent fashion [1]. Given that the glioprotective potential of IL-1 $\beta$  was established in this study, we wanted to examine if IL-1 $\beta$  could alleviate oxidative stress in neurons. Since, the brain has a high oxygen demand and is rich in lipids, it is vulnerable to injury by oxidants. Peroxides facilitate lipid peroxidation; hence we chose an organic peroxide, t-BOOH, a stressor previously used in several models of oxidative stress [166, 167].

Initial studies were devised to confirm previous findings of the laboratory, firstly that treatment of astrocytes with IL-1 $\beta$  increase in GSH production (Figure 3) [1]. This is in agreement with studies on non-neural cell types showing an association between IL-1 $\beta$  and GSH [168, 169]. However, in rat astrocytes, intracellular levels of GSH were shown to decrease following IL-1 $\beta$  exposure [170]. Similar results were demonstrated in a human glioma cell line [171]. The depletion of GSH in these studies was attributed to the production of oxidative stress occurring secondary to IL-1 $\beta$  treatment [170, 171]. In contrast, using the GSH:GSSG ratio as a proxy for the cells redox potential/oxidant status [172] as well as direct measurement of ROS, our laboratory found that IL-1 $\beta$  does not contribute to oxidative stress in murine astrocytes [1]. Overall, GSH levels in astrocytes can be regulated at multiple levels either affecting the enzymes, substrates or transporters [131].

Secondly, we confirmed that the increase in GSH is accompanied by glioprotection when IL-1 $\beta$ -treated astrocytes are exposed to t-BOOH (Figure 4) [1]. GSH has been reported to protect astrocytes against various insults [173-175].

Thirdly, the enhancement of GSH and consequent glioprotection is occluded in the absence of IL-1R1 (Figure 5) [1]. This indicates that our IL-1 $\beta$  preparation is devoid of contaminants (e.g., LPS) and that the observed effect is exclusively due to IL-1 $\beta$  signaling through the IL-1R1.

Finally, the fact that MK-571 concentration-dependently hindered the release of GSH from astrocytes under both basal and IL-1 $\beta$ -stimulated conditions (Figure 6A) suggests that Mrp1 transporter is involved in the GSH release, as has been demonstrated previously [1, 144, 155]. We had hypothesized that block of GSH release would prevent the glioprotective effect of IL-1 $\beta$ . However, the toxicity of t-BOOH and the protection afforded by IL-1 $\beta$  pre-treatment was not appreciably affected by MK-571 (Figure 6B).

The ability of IL-1 $\beta$  to enhance GSH production and to protect astrocytes in isolation from oxidant injury, did not extend to neurons cultured alone (Figure 7, 9 and 10). Interestingly, other studies have shown cultured neurons to have a bivalent response to IL-1 $\beta$ . For instance, Rothwell and Strijbos showed that IL-1 $\beta$  in the range of 0.5-1  $\mu$ g/ mL is neuroprotective against NMDA-induced toxicity; however, in the range of 10-100  $\mu$ g/ mL, it exacerbates injury in rat neuronal cell cultures [63].

The data suggested that IL-1 $\beta$ -stimulated astrocytes but not neurons make GSH and are protected against oxidative stress and this might not be surprising considering that recycling of GSH and its constituents between the astrocytes and neurons has been deemed important for antioxidant homeostasis in the CNS [142, 176]. It has been demonstrated that neuronal GSH levels are lower than astrocytic GSH [124] rendering them more susceptible to oxidative injury. It was found that neurons co-cultured with astrocytes have higher levels of glutathione than neurons cultured alone, suggesting that astrocytes enhance the antioxidant

levels [143]. Astrocyte-conditioned medium has a high amount of extracellular GSH. The dipeptides CysGly and  $\gamma$ -GluCys are products of GSH breakdown and they can serve as precursors for glutathione synthesis in the neurons. Cysteine is a rate-limiting substrate for GSH synthesis and it has been shown to be derived from astrocytes for the neuronal GSH homeostasis [145, 146]. Zhang *et al* showed that bone marrow stromal cells modulate the redox status of chronic lymphocytic leukaemia (CLL) cells and promote cellular survival by beneficial interaction leading to enhanced GSH synthesis [177]. We proposed that a similar interaction may take place between astrocytes and neurons in our model.

This led to investigation of response of mixed cultures to IL-1 $\beta$  treatment. We determined the GSH status of mixed cultures after IL-1 $\beta$  pre-treatment to find that there is enhancement in the extracellular GSH congruent with the results derived from astrocytes (Figure 11). Enhancement of GSH coincides with protection against t-BOOH-induced stress (Figure 13) that can be abrogated by inhibiting Mrp1-mediated GSH release by astrocytes using MK-571 (Figure 16). This pinpoints towards the synergy between astrocytes and neurons to maintain antioxidant homeostasis. Others have determined IL-1 $\beta$  to protect against excitotoxins in mouse mixed cortical cell cultures. Here they attribute the protection to the production of Nerve Growth Factor, but never looked at GSH levels or its potential to contribute [178].

Additional studies in slice culture demonstrate that IL-1 $\beta$  at similar concentrations to those used herein (10 ng/ mL) protected neurons against excitotoxicity and

oxygen/glucose deprivation-induced neuronal cell death [65]. Studies by Bernardino *et al* have also shown the biphasic effect of IL-1 $\beta$  by using mouse hippocampal slice cultures. It was shown that low dose (1 ng/ mL) of IL-1 $\beta$  potentiated AMPA-induced toxicity, whilst high dose (10 ng/ mL) of IL-1 $\beta$  significantly reduced the AMPA-induced toxicity [179]. *In vivo*, in a transgenic mouse model of Alzheimer's disease, 4 weeks of induced overexpression of IL-1 $\beta$  in the hippocampus reduced amyloid pathology also suggesting a protective role [4].

PI staining enabled us to qualitatively check for injury and neuroprotection in mixed cultures. Both neurons and astrocytes in t-BOOH-treated wells were stained with PI, establishing that both the cell-types in mixed culture were susceptible to oxidant injury (Figure 13). Further studies involving double immunolabeling for markers selective for neurons and astrocytes in mixed culture could help compare the extent of neuroprotection rendered by IL-1 $\beta$  to each type of cell.

Once we deciphered neuroprotection in mixed culture, we wanted to study any cell-specificity of response to IL-1 $\beta$  treatment of mixed cultures. Chimeric cultures were used for this purpose (Figure 14). Neurons when plated on *il1r1* *+/+* astrocytes but not on *il1r1* *-/-* astrocytes showed an increase in GSH, as we expected. This supported the hypothesis that astrocytes form the basis of the observed neuroprotection in co-cultures. Chimeric co-cultures were then challenged with 1.5 mM t-BOOH and it was observed that the presence of



astrocytic signaling is required for protection against cell death (Figure 15).

Interestingly, you will note that the viability of MTT assay appears slightly lesser when compared to the LDH readings. This is likely because the MTT assay requires incubation for at least two hours until the formazan crystals are formed (allowing for ongoing cell death) whereas LDH samples are read out immediately after termination of the experiment [153]. Although not shown in this thesis, others have determined that astrocyte-mediated support to neurons is lost upon glutathione depletion, again highlighting, the intricate interaction between neurons and astrocytes [123, 180, 181].

Lastly, we wanted to employ a model that mimics astrocyte-neuron redox coupling but allows for studying neurons in isolation to confirm whether GSH is the sole mediator of observed protection. As such, we set out to determine whether supplementing neurons in culture with GEE could substitute for astrocyte-mediated enhancement of GSH levels. GEE has been shown to increase cellular GSH, circumventing the regulation of GSH biosynthesis [182]. Other have demonstrated that 0.5 mM GEE protects neurons from amyloid  $\beta$  toxicity (10  $\mu$ M, 24 h) when pre-treated for one hour [183]. GEE (1–10mM, 24h) produced a dose-dependent elevation in GSH in rat mesencephalic cultures. The same study also showed that administration of 0.1–50mg/kg/d GEE to rats for 28 days, either subcutaneously or via an implanted cannula, up-regulated the GSH levels in the brain and protected neurons in a Parkinson's disease model [182]. GEE has been shown to be rapidly taken up and converted to GSH by tissues such as kidney, liver, spleen, pancreas and heart [184]. In our experiment,

treatment of the cultures with 0.3 – 3 mM GEE for 15 h, resulted in a significant increase in the intracellular GSH at 3 mM only (Figure 17A). Unfortunately, toxicity was observed at concentrations of 3 mM; and no effect on cell viability against t-BOOH- mediated stress was observed with the lower, non-toxic concentrations (Figure 17B). Hence, we cannot infer much from this model under the given paradigm.

Overall, our findings suggest that neuroprotection against t-BOOH in the given model may be dependent on GSH and/or other factors provided by the astrocytes to neurons. Astrocytes release a variety of trophic factors under normal conditions that assist neuronal survival and conduction after injury to the brain [185]. Future studies involving transwell co-culture of astrocytes and neurons aimed at studying the effect of IL-1 $\beta$  observed in mixed culture would provide direct evidence for whether neurons require physical contact with astrocytes for enjoying neuroprotection. Transwell co-culture can be recruited to study the exchange taking place between astrocytes and neurons that may be aiding neurons against oxidative damage.

In conclusion, this study confirms that IL-1 $\beta$  is neuroprotective and brings about an increase in glutathione in astrocytes and mixed cultures; however, the protection observed along with a concomitant increase GSH, necessitates the presence of astrocytes and a functional astrocytic IL-1R1 receptor. Interestingly, the lab has studied dichotomy in the action of IL-1 $\beta$ . We can therefore infer that the final outcome may protective, neutral or inimical, depending on but not limited

to its site of release, concentration, the environmental niche of the cells and the effector cell [165]. A better understanding of the physiological and pathophysiological consequences and the underlying mechanisms of IL-1 $\beta$  signaling in the CNS may serve the emergence of strategies to utilize its reparative potential.

## Chapter 5. Future Directions

We deciphered the neuroprotective potential of IL-1 $\beta$  signaling that was found to be localized to astrocytes. Along with t-BOOH, it would be interesting to assess neuroprotection against other stressors such as iron [186] and malonate [187].

Intracellular iron potentiates oxidative toxicity. Malonate leads to striatal specific injury and has been used as a model of Huntington's disease. Thus far, we have studied cortical cell cultures *in vitro*; however, it would be reasonable to test the response of striatal cell cultures to IL-1 $\beta$  and malonate injury. This would be beneficial in comparing results from cortical as well as striatal regions of the brain.

We have successfully demonstrated IL-1 $\beta$ -mediated protection via cell viability assays and qualitatively through PI staining. A quantitative analysis of markers of oxidative stress in mixed cultures would add to our existing findings. A previous study in the field has shown that t-BOOH treatment of rat hepatocytes causes generation of malondialdehyde and 8-hydroxydeoxyguanosine (8-OHdG) which are markers of oxidative stress [188]. These could be quantified in models involving mixed culture to complement results from cell viability assays.

Analysis of the astrocyte-conditioned medium can also help assess whether astrocytes release precursors for GSH synthesis in neurons. Another study that would corroborate our findings would be qRT-PCR (Real-Time Quantitative Reverse Transcription PCR) for the enzyme GSH peroxidase after IL-1 $\beta$  stimulation of astrocytes and neurons individually in culture.

Additionally, it would be interesting to study *in vivo* models addressing similar questions. The hypothesis that IL-1R1 null animals are more susceptible to oxidative injury than wild type control animals could be tested and findings be compared to the results from *in vitro* experiments. Previous studies have shown a strong linkage between excitotoxicity and oxidative stress which co-appear in several CNS disorders. NMDA is an excitotoxin known to increase oxidative stress. Model of NMDA-induced oxidative stress could be used for this aim and the role of endogenous IL-1 $\beta$  in conditions of oxidative stress could be assessed. Intra-cortical NMDA microinjections would enable us to carry out histological analysis and assess the extent of injury between *il1r1*  $+/+$  and  $-/-$  animals. Similarly, *in vivo* malonate models could be employed to study the hypothesis that IL-1R1 null mice are more susceptible to *in vivo* oxidative stress-mediated neurodegeneration. Behavioral studies such as Open field test [189] and rotarod test [190] would add another dimension what has been studied so far.

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| M.S. Biotechnology | 2012 | University of Mumbai, Mumbai, India<br>Thesis title: <i>In vitro</i> Interaction Studies between Flagellar Associated Protein 174 (Fap 174) and Radial Spoke Protein 3 (Rsp 3) from the green chlorophyte <i>Chlamydomonas reinhardtii</i> .<br>Advisor: Dr. Jacinta D'Souza<br>(UM-DAE Centre for Excellence in Basic Sciences) |
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